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14. ABSTRACT
Increased genomic instability arising from centrosomal amplification has been proposed to be an important factor causing development of traits associated with highly malignant ovarian tumors, including multidrug resistance and increased tendency to metastasis. This proposal addresses the hypothesized interaction between the Cas proteins (HEF1 and p130Cas), Aurora A (AurA) and Ajuba as being likely to contribute to genomic instability and metastatic properties of ovarian tumors. In this proposal, we examine tumor samples to determine if Cas expression, activated AurA, and centrosomal amplification are linked, and whether Cas protein upregulation is associated with a poor prognosis (Aim 1). We examine the mechanism by which Cas proteins activate AurA, and determine if drug-mediated inactivation of AurA inhibits Cas promotion of aneuploidy (Aim 2). We use drug and depletion experiments to determine if centrosome amplification and enhanced cellular metastasis are linked, and dependent on Cas/integrin signaling, or whether these are separable properties; and to evaluate combination of AurA- and integrin- directed therapies (Aim 3). In this annual report, we describe significant process on all Aims that validate the hypothesis of critical AurA-HEF1-Ajuba interactions.

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INTRODUCTION: The goal of this proposal is to better understand factors leading to ovarian cancer development, with the intent of improving diagnosis and treatment for ovarian cancer. Our preliminary data defined the HEF1 member of the Cas protein family as a regulator of centrosomal dynamics and genomic instability through control of the Aurora A (AurA) kinase. The goal of the proposal was to explore HEF1 and p130Cas protein status as a contributing factor to early onset of ovarian cancers, and use this information to assess the value of combining targeted small molecule therapeutics for ovarian cancer therapy. Three Aims were proposed to address this objective. In *aim 1*, we proposed to examine tumor samples to determine if HEF1 or p130Cas expression, activated AurA, and centrosomal amplification are linked, and whether Cas protein upregulation is associated with a poor prognosis. In *aim 2*, we proposed to examine the mechanism by which Cas proteins activate AurA, and determine if drug-mediated inactivation of AurA inhibits Cas promotion of aneuploidy. In *Aim 3*, we proposed to use further drug and depletion experiments to determine if centrosome amplification and enhanced cellular metastasis are linked, and dependent on Cas/integrin signaling, or whether these are separable properties; and to evaluate combination of AurA- and integrin- directed therapies.

BODY: During the last reporting period, we have made significant progress on all aims, resulting in two publications (discussed below) and more works in preparation.

Aim 1: Determination of whether HEF1 or p130Cas expression, activated AurA, and centrosomal amplification are linked, and whether Cas protein upregulation is associated with a poor prognosis. In this Aim, we first proposed to use Western blot analysis of tumor lysates to correlate expression of HEF1, p130Cas, Aurora A, and phospho-Aurora A. This analysis is in progress. In the first several months of the study, we optimized choice and hybridization conditions for antibodies, harvest conditions for tumor cell lysates, and importantly, evaluated a number of different detection/image analysis systems to ensure reproducible quantitative results. We have used a pilot set of 48 ovarian tumors. Each tumor lysate has been run out and visualized in at least two independent experiments, with three different concentrations of two reference cell lines (human ovarian surface epithelium (HOSE) and MCF-7 (a breast adenocarcinoma cell line that expresses abundant HEF1 and p130Cas) present on every gel as internal control. All samples are reprobed with actin to confirm even loading, and data are analyzed based on actin normalization. We have compiled data using both NIH Image to quantify scanned X-ray films and an LAS-1000 (Fujifilm), and are now in the process of shifting to the Odyssey LI-COR Biosciences system, which allows detection of two different signals simultaneously in a single exposure that is automatically quantitated and corrected for loading variance. Based on these optimizations, data generation is moving smoothly and is highly reproducible

Interestingly, preliminary statistical analysis using Spearman and Pearson correlation indicates at least one striking correlation: overexpression of HEF1 inversely correlates with total levels of AurA. This was unexpected based on our model, but the finding is very significant ($r = -0.85$, $P < 0016$ for Spearman correlation) in this set of tumors. Intriguingly, although levels of total AurA inversely correlated with HEF1 expression, levels of phosphorylated (activated) AurA did not, suggesting that AurA may

show greater specific activity in the presence of overexpressed HEF1: if validated by further analysis, this would be congruent with our model. We are now expanding the set of tumors analyzed, to include tumors with different classifications and with differing prognoses.

In parallel to this analysis, as proposed in Aim 1.2, we have analyzed by IHC a set of eleven tissue microarrays containing >220 ovarian tumors of various stages and histotypes with antibody to Cas proteins. We obtained both progression-free survival and overall survival for many of the tumor samples evaluated. The tumors were scored on a scale of 0 to 4, zero being no detectable signal and for being highly positive for Cas. We did not find a positive correlation between IHC scores and survival using different cutoffs in high-grade carcinoma. However, we did observe a potentially interesting relationship between high-grade and low-grade micropapillary serous carcinoma (MPSC) if we use 0-2 and 3-4 as groups ($p = 0.013$). Although ovarian cancer is often viewed as a single disease, it is considerably more complex and represents a family of related but distinct tumors. Recent studies, although somewhat controversial, suggest that serous carcinoma comprises at least two distinctive types of tumors. The conventional type of serous carcinoma grows rapidly and kills patients within 5 years despite aggressive treatment, and the second type, designated MPSC, is low grade and indolent but fails to respond to conventional chemotherapy. The molecular basis that distinguishes CSC and MPSC is unknown, but potentially important to rational development of early diagnostic tests and effective, specific therapy. In our studies if appears that a higher percentage of low-grade cases of MPSC have strong Cas staining (3-4), however, our set had only a limited number of these cases. In additions, we consistently observe strong Cas staining in tumors of low malignant potential. This observation will need to be further explored using additional samples.

Aim 2: Determining the mechanism by which HEF1 and p130Cas proteins activate AurA, and whether inactivation of AurA inhibits Cas promotion of aneuploidy. This Aim had several sub-Aims: 1. Do HEF1 and AurA directly associate? 2. Does p130Cas associate with AurA? 3. Does addition of HEF1 or p130Cas to AurA induce the kinase activity of AurA in vitro? 4. Is HEF1 itself is an AurA substrate? 5. Does HEF1 associate with Ajuba? 6. Does inhibition of the AurA kinase inhibit the centrosomal amplification and aneuploidy induced by HEF1 overexpression?

We have made exceptional progress on this Aim, which built on the preliminary data present at the time the proposal was submitted. We have completely addressed points 1-5. Detailed description of points 1-4 have been published in two manuscripts(*1*, *2*) (see Appendix). HEF1 and AurA interact, with HEF1 required for AurA activation; p130Cas does not interact with AurA. HEF1 is an AurA substrate; phosphorylation of HEF1 by AurA causes the two proteins to bind each other with lower affinity. The biological significance of these findings is discussed at length in the provided manuscripts. For point 5, we have shown that not only do HEF1 and Ajuba interact, but also HEF1 and AurA synergize to induce AurA activity (see *Figure 1*). We will address point 6 in the next funding period.

Aim 3: Determination of whether if centrosome amplification and enhanced cellular metastasis are linked, and dependent on HEF1/integrin signaling, and whether

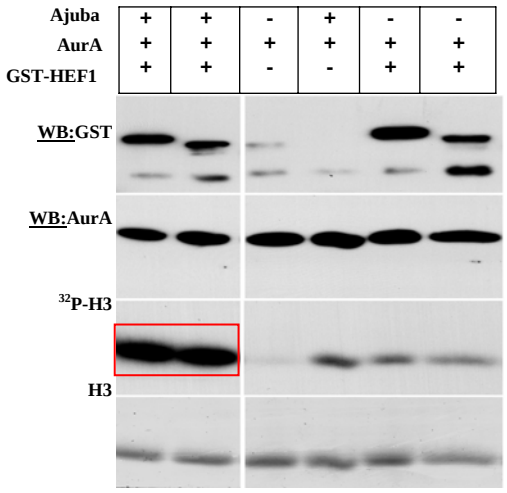

Figure 1. In vitro expressed AurA, Ajuba, and GST-HEF1 (two related N-terminal derivatives of HEF1) were combined as indicated with histone H3. AurA kinase activity was measured using an *in vitro* kinase assay based on incorporation of ^{32}P-ATP into histone H3. Box emphasizes synergistic activation of AurA in reactions containing both GST-HEF1 and Ajuba.

combination of integrin-pathways targeted inhibitors with AurA inhibitors is of clinical merit. This Aim had the following sub-Aims: 1. We will perform structure-function analysis to evaluate whether HEF1 control of cell attachment, and regulation of centrosome-associated functions utilize overlapping or separable domains. 2. We will determine if inhibition of HEF1 signaling by inhibitors targeting the integrin pathway blocks HEF1 ability to induce centrosomal amplification. 3. We will determine whether inhibition of AurA signaling blocks HEF1-dependent cell migration and invasion. 4. We will determine whether combination of AurA- and integrin-targeted inhibitors synergistically blocks both cell migration/invasion, and centrosomal amplification.

We have completely addressed sub-Aim 1, with this data included in the publication supplied: different domains of HEF1 are required for the two functions (*1*). We are currently engaged in the remaining studies shown in this Aim, and hope to complete this work over the next year.

KEY RESEARCH ACCOMPLISHMENTS:

- We have optimized conditions for screening tumor lysates by Western blot for expression of all the proteins noted in the Aims.
- We have identified a statistically significant negative correlation between HEF1 overexpression and Aurora A overexpression.
- We have begun to analyze HEF1/AuroraA/centrosome expression in tissue microarrays.
- We have demonstrated a critical interaction between HEF1 and Aurora A that is necessary for Aurora A activation and mitotic progression.
- We have demonstrated that HEF1 interacts with Ajuba, with HEF1 and Ajuba synergizing to activate Aurora A.
- We have mapped the domains of HEF1 required for action at centrosome and focal adhesions, and we have shown that these are separable.

REPORTABLE OUTCOMES:

To date, this project has resulted in two manuscripts that have been published in Nature Cell Biology and in Cell Cycle. A third manuscript, describing HEF1-Aurora A- Ajuba interactions, is currently in preparation. Data from this project has been presented this past July at the FASEB Conference on Rho GTPases, and at an invited seminar at

University of North Carolina- Chapel Hill, and will be included at two posters to be presented in December 2006 at the annual meeting of the American Society for Cell Biology. Data from this project has been used to support a grant application to the NIH for an unexpected extension of our results in the area of polycystic kidney disease (see below).

CONCLUSION:

The study to date has validated a completely novel mechanism of activating Aurora A at the centrosome, through use of HEF1 and Ajuba. This extremely important finding connects cell adhesion and cell cycle signaling, and provides insight to the linked deregulation of these processes in cancer. We have expanded on these ideas in a recent review on this topic published this fall (*3*).

For further context, we would note two additional points describing work beyond the immediate scope of the proposal. First, this past summer, amplification- or transcription-based overexpression of HEF1 (also known as NEDD9) was identified as a major pro-metastatic factor for melanoma (*4*). Overexpression of HEF1 has also been found as part of a pro-metastatic signature in breast adenocarcinoma (*5*), and we have collaborated with the Gladson laboratory to show that HEF1 contributes in an important way to the invasive behavior of glioblastomas (*6*). Second, building from our identification of HEF1 as an activator of Aurora, we have now defined a HEF1-Aurora A signaling switch as a major determinant of ciliary disassembly. The abstract of a paper we have recently submitted on this topic reads as follows:

> "The mammalian cilium protrudes from the apical/lumenal surface of polarized cells, and acts as a sensor of environmental cues, including physical stimuli such as directional fluid flow, and diffusible. Numerous developmental disorders and pathological conditions have been shown to arise from defects in cilia-associated signaling proteins. Despite mounting evidence that cilia are essential sites for coordination of cell signaling, almost nothing is known about the cellular mechanisms controlling their formation and disassembly. Here we define a novel signaling pathway in which interactions between the pro-metastatic scaffolding protein HEF1/Cas-L/NEDD9 and the oncogenic Aurora A (AurA) kinase at the basal body of cilia causes phosphorylation and activation of HDAC6, a tubulin deacetylase, promoting ciliary disassembly. We show that this pathway is both necessary and sufficient for ciliary resorption, and constitutes a novel, non-mitotic activity of AurA in vertebrates. Moreover, we demonstrate that small molecule inhibitors of AurA and HDAC6 selectively stabilize cilia from regulated resorption cues, suggesting a novel mode of action for these clinical agents."

Based on these studies, we believe that HEF1-AuroraA interactions will turn out to be extremely important for coordinating signaling not only at focal adhesions and the centrosome, but also at cilia; and that HEF1 may constitute a point of vulnerability in cancer because of these many connections. The conclusion of the present studies will help provide the justification for combined use of clinical agents targeting Aurora A, integrins, and potentially histone deacetylases as tools to combat cancer.

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APPENDICES:

SUPPORTING DATA: N/A (Figures embedded in text).

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ERICA A. GOLEMIS, Ph.D., PRINCIPAL INVESTIGATOR

APPENDIX

Publications Resulting from this Grant

1. Pugacheva, E.N., Golemis, E.A. HEF1-aurora A interactions: points of dialog between the cell cycle and cell attachment signaling networks. *Cell Cycle* **5**(4):384-391, 2006, Review.

2. Pugacheva, E.N., Golemis, E.A. The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. *Nat. Cell Biol.* **7**(10):937-946, 2005.

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Perspective

HEF1-Aurora A Interactions

Points of Dialog Between the Cell Cycle and Cell Attachment Signaling Networks

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KEY WORDS

centrosome, polarity, mitosis, attachment, cilia, Aurora-A, HEF1, Cas

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ABSTRACT

Regulated timing of cell division cycles, and geometrical precision in the planar orientation of cell division, are critical during organismal development and remain important for the maintenance of polarized structures in adults. Mounting evidence suggests that these processes are coordinated at the centrosome through the action of proteins that mediate both cell cycle and cell attachment. Our recent work identifying HEF1 as an activator of the Aurora A kinase suggests a novel hub for such integrated signaling. We suggest that defects in components of the machinery specifying the temporal and spatial integration of cell division may induce cancer and other diseases through pleiotropic effects on cell migration, proliferation, apoptosis, and genomic stability.

INTRODUCTION

During metazoan development, cell division is regulated by diffusible and localized extracellular factors that promote or inhibit proliferation, specify mitotic division orientation and symmetry, regulate differentiation into distinct cell types, and in some cases promote directed migration or apoptosis of dividing cells. These cues are essential during the conversion of a single fertilized egg into a complex multicellular organism. They remain important in adults, coordinating the limited cell division required for maintenance of organs. Because of these critical regulatory roles, mutated forms of the proteins comprising the machinery to transmit extracellular information to the cell division apparatus are frequently identified as oncogenes and tumor suppressors, or as cancer-predisposing factors. As summarized below, work by many groups has begun to outline a network of signaling proteins that operate to connect these processes, many of which utilize the centrosome as a central communication point for transmission of information. Aurora-A (AurA) kinase[1,2] is now appreciated as an important transducer of signals at centrosomes; our recent studies describing interactions between HEF1 and AurA required for AurA activation[3] illuminate a new branch of this signaling network. In this article, we will first summarize the diverse signaling functions that have been identified for centrosomes, then describe how the association of AurA and HEF1 may impact these functions.

ROLES OF THE CENTROSOME

The centrosome is composed of two paired orthogonal centrioles surrounded by "pericentriolar material" (PCM) that varies in abundance and content during cell cycle, and comprises hundreds of structural and signaling proteins. The centrosome has its own duplication cycle (reviewed in refs. 4–7), and was for a long time thought of predominantly as an organizing structure for cellular microtubules (a microtubule organizing center, MTOC). As such, its actions in physically nucleating the two ends of the mitotic spindle were a major focus of study. Through studies over the past decade, this view of the centrosome has been significantly revised. It has now been shown that the centrosome provides a contained platform to coordinate signaling related to polarity and cell cycle coordination. Several excellent reviews summarize centrosomal biology at length.[7-10] In brief (see also Fig. 1), important centrosome-associated functions to consider include:

(1) Orientation of the mitotic spindle in asymmetric cell divisions. Cells growing in a plane (for example, as a sheet of epithelial cells) may divide in different directions. Symmetric planar division can extend the size of the sheet, with two daughter cells assuming the same fate as their mother. Asymmetric cell divisions orthogonal to the direction of the plane allow a mother cell to spawn two daughter cells with different cell fates, and can

cause cell propagation into a new dimension. Drosophila has been a productive model system for demonstrating the importance of the centrosome in these processes. Recent studies have addressed asymmetrically dividing neuroepithelial cells giving rise to neuroblasts,[11,12] male germline stem cells producing gonialblasts after an apical-basal division away from a germline stem cell "hub"[13] (Fig. 1A), and the syncitial divisions of early embryos.[14] In these works, centrosomes have been shown to be the target for proteins that directly orient the mitotic spindle by forming physical bridges with polarity cues associated with the cell surface and cortical actin. Planar (lateral) divisions are specified based on signals from the ademotous polyposis coli (APC) tumor suppressor protein, and Armadillo/beta-catenin. In the absence of these dominant signals, basal signals provided by Bazooka/Par3, a component of the cell polarity machinery,[15,16] can direct cell divisions along the apical-basal axis.[11]

There is mounting evidence that this signaling machinery is conserved through evolution. APC is distantly related to Kar9p, an *S. cerevisiae* protein that acts as a cue for orientation of the mitotic spindle to the bud, and associates with both the spindle pole body (the yeast "centrosome") and the actin cortex within the tip of a forming bud.[17,18] Excitingly, recent studies by Lechler and Fuchs have provided evidence for a similar process occurring during the stratification and differentiation steps of epidermal development in mammals.[19] In this case, both integrins and cadherins provided essential signals regulating the polarity complex (Par3 /Pins/aPKC), and additionally influence NuMA and dynactin activity, thus controlling the orientation of the centrosome and spindle.

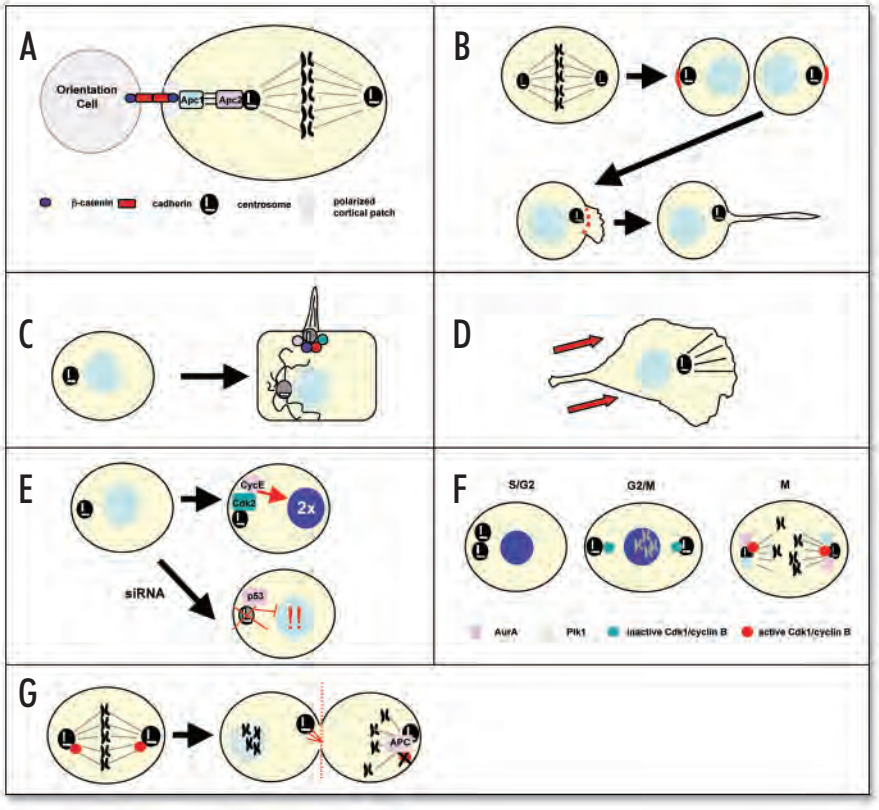

Figure 1. The centrosome coordinates diverse cellular processes. These include (A) orientation of the mitotic spindle in asymmetric cell divisions, (B) specification of the site of neurite process extension (neurites), (C) nucleation of cilia and flagella, (D) orientation of cell migration, (E) and regulation of cell cycle progression through G_1 (checkpoints), (F) mitotic entry, and (G) mitotic exit. See text for details.

(2) Specification of the site of process extension (neurites and cilia). Centrosomes also influence the polarization of external cell processes. In a recent study of neurite formation that utilized both mammalian hippocampal neurons and Drosophila third instar neurons,[20] it was shown that formation of a neurite projection from an apparently undifferentiated, rounded cell body occurred at the site where the centrosome and associated Golgi apparatus abut the cell cortex (Fig. 1B). This site is specified based on the prior mitotic division, such that the new neurite forms on the opposite side of the cell from the previous cleavage plane. After cytokinesis, the cortex proximal to the centrosome undergoes transient lamellipodial extension. This is followed by formation of what becomes the dominant neurite at the same site. The observation that an actin polymerization inhibitor (cytochalasin D) can suppress neurite extension implies initial reorganization of the actin cytoskeleton precedes the organization of microtubules and secretory machinery at the time of neurite extension.

As a separate example of centrosome-based polarization of non-mitotic structures, in nonproliferating (G_0, stationary) eukaryotic cells, one of the centrioles within a centrosome undergoes a differentiation to form the ciliary basal body (ref. 21 and refs. therein), which then recruits microtubules and the vesicular trafficking machinery to create a cilium (Fig. 1C). These cilia are non-motile, share many (although not all) proteins with centrosomes, and are typically reabsorbed if cells return to proliferation. Mutations in a number of the proteins associated with basal body (for example, inversin and others) are associated with diseases involving abnormal planar cell polarity, and in some cases, abnormal cell growth (refs. 10, 22 and others). Simons et al. have shown that inversin, a cilia-basal body-centrosome protein, directly interacts with Disheveled, which associates in turn with β-catenin,[23] previously shown to orient the spindle in *Drosophila* studies.[13] Increasing evidence suggests that in stationary cells, receptors for external signals are specifically localized to the cilia.[24] It has been proposed that the cilia may also coordinate signals determining whether cells remain in or emerge from stationary phase, through communication with the cell cycle.[25] In this context, the Nek kinase family may play an important role, as many of the members of this family of kinases are distributed between basal body and centrosome, and some (e.g., Nek2) are known to regulate centrosome dynamics and possibly affect spindle checkpoints, through influencing centriolar cohesion.[21,26]

(3) Centrosomes and cell migration. The studies of centrosomes in neurites suggesting a role for centrosome in regulation of both actin and tubulin cytoskeletons is of additional interest because of reports suggesting a role for the centrosome in orienting cell migration (Fig. 1D). An initial study in Dictyostelium observed that positioning of the centrosome in front of the nucleus, behind the leading edge of a migrating cell, was important for the stabilization of the direction of cell migration, perhaps by orienting the microtubule network in support of the actin-based motility machinery.[27] Subsequent work by others in some cases supported,[28] and in others contradicted this

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observation,[29] while additional work has suggested that the contribution of the centrosome may be to increase the efficiency rather than directionality of migration, through regulation of microtubule dynamics.[30] In one model, of fibroblast migration, it has been demonstrated that a signal dependent on the Cdc42 GTPase is required to orient the centrosome to face the direction of migration, while microtubules emanating from the centrosome interact at their plus ends with EB1 and APC, making contact with the cell cortex at sites involving Discs-large (Dlg), and once again specified by the polarity complex (aPKC and other proteins).[31-33] At present, it seems likely that the phenomenon of centrosomal contribution to migration is cell type specific,[34] which may reflect the abundance of differing polarization-associated proteins in diverse cell types. It is only now becoming widely appreciated that cells migrate by a variety of different strategies, and that as cancer cells become metastatic, they can serially adapt different strategies:[35,36] hence, the importance of these observations in human disease remains to be established.

(4) **Requirement for progression through G_1 (checkpoints).** Centrosomal integrity is important for a number of different cell cycle transitions. Cells with centrosomes ablated by multiple approaches (ref. 9, and Refs. therein) undergo G_1 arrest (Fig. 1E). One mechanism proposed for this arrest is based on the observation that the cyclin E/Cdk2 and cyclin A/Cdk2 complexes that promote entry into S phase have an obligate association with centrosomes,[37,38] such that cyclin E mutated to eliminate a centrosome localization domain is unable to promote entry into S phase.[37] Conversely, Cdk2/cyclin E also is required for the centrosome duplication cycle,[38,39] and overexpression of cyclin E can promote centrosome overduplication.[37] A second mechanism of centrosome control of cell cycle may involve the activation at the centrosome of a p53-dependent cell cycle checkpoint, as cells with defective centrosomes (due to depletion of components by siRNA) do not undergo G_1 arrest in p53-deficient cells (and discussed in refs. 9 and 40).

(5) **Roles in entering and exiting mitosis.** The best-studied aspects of centrosomes are their roles in relation to G_2/M processes (Fig. 1F; reviewed in depth in refs. 5, 7-9 and 41). Prior to G_2/M transition, a series of interactions between Cdk1 and inhibitors such as Chk1 and Cdc25B at the centrosome restrain Cdk1 activity. At mitotic entry, the AurA and Plk1 kinases act at the centrosome to activate Cdk1/cyclin B and perform other actions necessary to initiate the intracellular organization accompanying karyo- and cyto-kinesis.[42,43] Gamma-tubulin and other proteins associate with PCM components such as pericentrin, promoting formation of astral microtubules.[44] Later in mitosis (Fig. 1G), centrosomes are centers for ubiquitination activity, governing the action of the anaphase promoting complex/cyclosome (APC/C) in causing the degradation of substrates such as cyclin B.[45-47] Separate studies indicate that the centrosome may also nucleate cellular degradation and proteasome activation at other phases in the cell cycle as well.[48] In 2001, Piel et al. made the intriguing observation that the mother centriole must undergo an excursion to the region of the midbody to allow completion of cytokinesis, suggesting delivery of some final signal to promote excision.[49] Although the nature of this signal remains to be established in detail, Cep55, which interacts with ERK kinases as well as Cdk1 and Plk1, has recently been shown to migrate from centrosome to midbody at cytokinesis, and play an important role in abscission and return to G_1.[50]

INTRODUCTION TO AurA

The AurA kinase is also known as STK15, STK6, BTAK, ARK1, HsAirk1, and Aik; it is a member of the Ipl family of kinases (reviewed in ref. 1). AurA is abundant at the centrosome in G_2 to M phase, degraded upon completion of cytokinesis, and present at very low levels in G_1 and S phases, in part because of efficient post-translational degradation by the ubiquitination machinery.[51] Although present at the centrosome from early G_2, AurA only becomes active around the time of prophase. This activation process is not completely understood, but requires AurA interactions with the proteins Ajuba,[42] TPX2,[52,53] and (as we have recently described ref. 3) HEF1. Upon activation, AurA phosphorylates substrates that promote progression through the stages of mitosis: these include Cdc25B, TPX2, Eg5, Lats2, histone H3, D-Tacc, Brca1 and others, with the list continuing to expand (reviewed in ref. 1). Among its defined activities, one of the most important is in promoting the activation of cyclin B/Cdk1,[42] which occurs physically at the centrosome, and may be mediated through phosphorylation and inactivation of Cdc25B.[54] Failure of AurA activation results in G_2 arrest or a defective entry into mitosis, marked by failure of centrosomes to separate and associated monopolar mitotic spindles, and consequent defects in chromosome alignment: failure to complete cytokinesis may arise from this, or also involve additional defects.[55,56]

In the past several years, AurA has attracted increasing attention because it has been found to be overexpressed in many tumors arising from breast, colon, ovary, and other tissues,[57-61] and because it has been shown to function as an oncogene when exogenously expressed in various cell line models.[62-65] AurA overexpression, whether in naturally occurring tumors or following deliberate overexpression, is associated with increased numbers of centrosomes and multipolar spindles, which arise as a consequence of failed cytokinesis. As the overexpressed AurA is not limited to expression in G_2 and M phases at the centrosome, but is also detecting throughout the cytoplasm in cells in all cell cycle compartments, it is not clear at present whether the transforming activity of AurA arises from hyperactivated AurA targeting its normal substrates, or through anomalous targeting by AurA of additional substrates (as in the refs. 61 and 66). Unexpectedly, even overexpression of a kinase-inactive form of AurA can induce supernumerary centrosomes (although it cannot transform cells),[62] supporting the idea that the protein has at least two different functions in regulating centrosome numbers. At least one set of important functions of the overexpressed active AurA is to override the spindle checkpoint, which causes resistance to spindle targeting agents such as taxol[63] and may arise in part through abrogation of the function of the Chfr mitotic checkpoint protein.[67] Separately, numerous reports have now documented a physical association between AurA and p53, most likely occurring directly at the centrosome (e.g., refs. 68-70). Although the functional consequences of these interactions are currently controversial, based on conflicting studies using varying assay conditions,[62-64,68,69] it appears that AurA is able to influence and in some cases override the post-mitotic checkpoint. Based on these various properties, AurA is now being actively exploited as a target for development of new anti-cancer agents (reviewed in ref. 2).

INTRODUCTION TO HEF1

The newcomer to the discussion of AurA and centrosome functions is HEF1 (ref. 71 also known as Nedd9 and Cas-L).[72] HEF1 and two related proteins, Efs/Sin,[73,74] and p130Cas/Bcar1,[75] comprise

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the Cas protein family.[76,77] These proteins are multidomain scaffolding proteins, with an amino-terminal SH3 domain followed by a large number of potential SH2 binding sites in a "substrate domain"; the carboxy-termini of the proteins, although well conserved within the family, are less well functionally characterized, lacking significant sequence homology outside the group. The first established and best-studied role for this group of proteins is as components of the integrin-dependent attachment signaling cascade, localized to focal complexes and focal adhesions on the basal cell surface. Upon receipt of attachment signals from the extracellular matrix through integrins at the focal adhesion, Cas proteins associate with focal adhesion kinase (FAK) and a Src family kinase. As a result of these interactions, the activity of Src is elevated,[78,79] and Src phosphorylates Cas extensively in the Cas substrate domain, creating active SH2 binding sites.[80] These sites bind the adaptor protein Crk/CrkII, subsequently recruiting DOCK180 and C3G; these associations cause signals to propagate further, through DOCK180 to Rac and Pak, and through C3G to the Ras-related GTPase Rap1, in each case promoting lamellipodia formation and cell migration.[81,82] HEF1, p130Cas, and Efs each increase cell migration when overexpressed.[76,77]

Extending out from this set of functions, members of the Cas family have also been shown to influence additional cell processes. Through the C3G-Rac signaling axis, p130Cas was shown to be important for phagocytosis.[83,84] In normal epithelial cells, detachment of a cell from external supports triggers a suicide program termed "anoikis", which acts as a surveillance mechanism against cancer.[85,86] Cas proteins are components of the attachment-dependent cell survival signaling cascade, with both HEF1 and p130Cas influencing cell viability under different attachment conditions.[87,88] Elevated Cas levels activate Ras-dependent pathways,[89] enhancing Raf>MEK>ERK proliferation signaling, and also stimulating PI-3-K.[90] P130Cas overexpression has been shown to confer tamoxifen resistance on cells, and elevated expression of Cas proteins has been shown to associate with poor prognosis in breast cancer, although the mechanism for Cas action in these cases is not well defined.[91-95]

Although the Cas proteins have many overlapping functions, some features distinguish HEF1. The most well-studied member of the Cas family, p130Cas, is near ubiquitously expressed. In contrast, HEF1 expression varies considerably between different cell types and tissues.[71,72,96,97] It is most abundant in vivo in tissues with polarized cell populations, including epithelial cells, neuronal and glial cells, and lymphoid cells, and its signaling action may be particularly important in these cell lineages.[97,98] p130Cas is abundant at all phases of cell cycle. In contrast, HEF1 is very low in G_0/G_1 phase cells, with abundance peaking in G_2 and M phase.[3,99] HEF1 expression is induced by various pro-growth or pro-migratory stimuli, including all-trans retinoic acid, which induces polarized neurite extension in brain development,[100] and TGF-beta,[101-104] which induces epithelial-mesenchymal transition in development and metastasis:[105,106] recently, HEF1(Nedd9) elevation was described as part of the lung metastasis transcriptional signature.[107] Besides being transcriptionally regulated by TGF-beta, HEF1 physically associates with downstream effectors of TGF-beta, the SMAD proteins: this causes post-translational regulation of the protein via the ubiquitination-proteasome machinery,[102-104] and raises the possibility that interaction with HEF1 may target other proteins for proteasomal degradation. Finally, we have now shown that HEF1 localizes to the centrosome, where it associates with and positively regulates the activity of AurA kinase, through a mechanism yet to be defined.[3]

AurA, HEF1, AND EXPANDED ROLES IN CENTROSOME-ASSOCIATED SIGNALING

In our recent study,[3] we demonstrated that like AurA, HEF1 accumulated at the centrosome predominantly between G_2 and M phase in normal and cancerous breast cell lines. Depletion of HEF1 by siRNA did not affect AurA accumulation at the centrosome, but blocked the activation of AurA at mitotic entry, and led to accumulation of cells with monopolar spindles. Conversely, overexpression of HEF1 induced AurA hyperactivation, and produced cells with multipolar spindles and supernumerary centrosomes. HEF1 also activated AurA kinase activity with both proteins in a purified in vitro system, indicating a direct mode of action. Further, in vitro and in vivo domain mapping experiments demonstrated that the sequences of HEF1 required for AurA activation differed from those required for HEF1-dependent regulation of cell spreading, ruling out the possibility that the HEF1-dependent effects at the centrosome seen were secondary consequences of changes in cell attachment. Finally, the phenotype of HEF1 differed in one important way from that of AurA depletion: in HEF1-depleted cells, premature splitting of the centrosomal pairs was observed, such that in an asynchronous population with similar profiles ~70% of cells had separated centrosomes, rather than ~27% in control siRNA-depleted cells.[3] This implied that HEF1 might have a second action at centrosomes, in regulation of centrosomal cohesion. Indeed, we showed that HEF1 negatively regulated the action of Nek2, such that this kinase, which promotes centrosome splitting,[26,108-110] had enhanced activity in HEF1-depleted cells. At present, it is not clear whether this reflects a direct or indirect consequence of loss of HEF1.

Upon initial inspection, the association of HEF1 and AurA, and the implicit potential for cross-signaling between the focal adhesion attachment machinery and centrosome-based cell division machinery, may seem surprising. However, returning to the list of centrosomal signaling roles summarized above, there are a number of reasons why the establishment of HEF1-AurA association is relevant to current models for development and cancer (Fig. 2).

First, as noted above, the orientation of cell division plane depends in part on both planar (cadherin-associated) and basal (integrin-associated) external adhesion cues. Focusing on basal signals, HEF1 and Pak, both of which are downstream integrin effectors in cell migration, are now known to associate with and activate AurA at the centrosome,[3,111] discussed in.[112] Pak centrosomal localization requires association with another centrosomal protein, GIT1, which also binds the focal adhesion protein paxillin.[113] Thess are not isolated instances of focal adhesion proteins finding a new use in mitosis. For example, the mitotic kinase WARTS/Lats has been shown to interact with the focal adhesion protein zyxin, with both proteins proximal to the centrosome at the astral microtubules in early mitosis, and collaborating in mitotic initiation (e.g., ref. 114). In their recent work describing extracellular matrix control of cell division axis, Thery et al. have proposed that one factor contributing to spindle orientation is cell shape anisotropy arising from greater membrane retraction on non-adhesive surfaces.[115] Interestingly, Cas and associated proteins CrkII and C3G have been implicated as an integrin-associated stretch-sensing machinery, with application of mechanical force activating downstream signaling.[116] The numerous connections between focal adhesions and centrosomes now being identified make it plausible that reuse of the existing basal attachment machinery in the G_2 and M phase of cell cycle may offer an economical means to coordinate mitotic division polarity.

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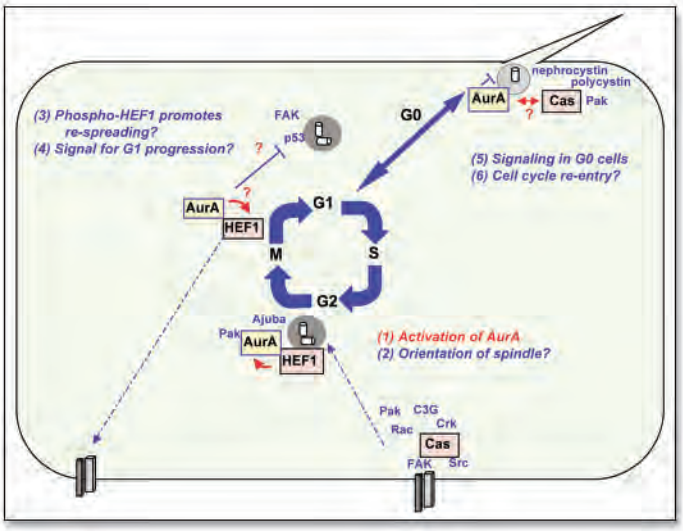

Figure 2. Demonstrated and speculative models for HEF1 and AurA interactions. We have shown (1) that HEF1 promotes AurA activation at the centrosome. Based on the biology summarized herein, and demonstrated protein-protein interactions placing AurA and HEF1 or p130Cas at specific intracellular locales, we speculate that HEF1 and/or p130Cas (2) may be a component of the integrin-dependent machinery orienting the mitotic spindle. We hypothesize that hosphorylation of HEF1 by AurA (3) may promote HEF1 localization to reestablishing focal adhesions at the end of mitosis, contributing to cell spreading: impairment of the HEF1-AurA interaction, or defective post-mitotic spreading, may contribute to activation of p53 and a post-mitotic checkpoint (4). Separately, HEF1 or p130Cas at cilia may coordinate signaling complexes in G_0 cells (5), or in response to external signals (growth reentry, or shearing force, given the hypothetical role of Cas proteins as stretch sensors, may trigger AurA activity, leading to ciliary disassembly (6). Proteins with which Cas or AurA have been shown to functionally associate relevant to these models are noted in blue.

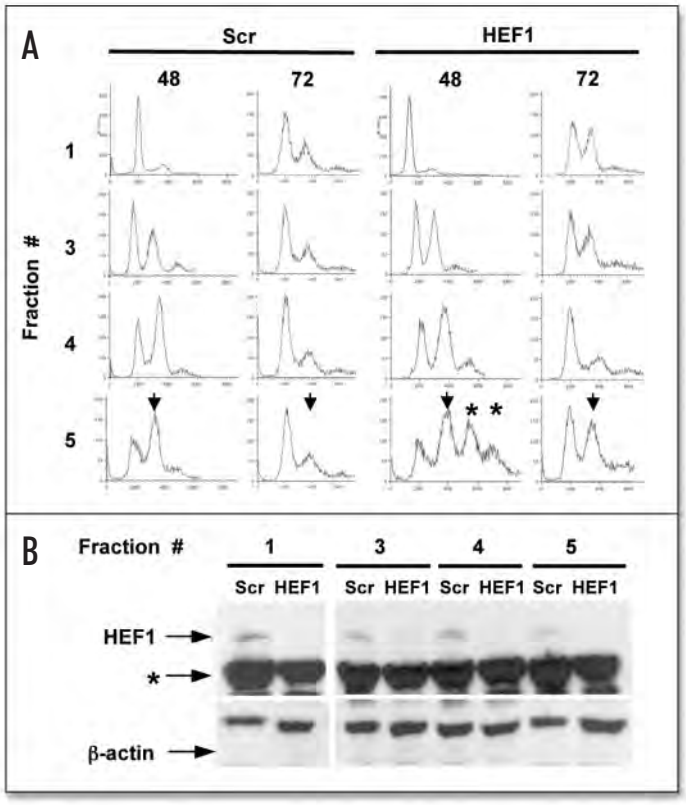

Figure 3. Depletion of HEF1 causes transient aneuploidy. (A) 10^9 MCF7 cells treated with control nonspecific siRNA duplex (Scr) or HEF1-specific siRNA for 48 h were elutriated to separate cell cycle fractions using a Beckman J elutriating centrifuge. The FACS profiles of representative fractions across the gradient are shown (48 hours): in parallel, an aliquot of each fraction of the elutriated cells was replated, grown for an additional 24 hours, and then reassayed by FACS (72 hours). Arrows in fraction 5 indicate 4N DNA peaks; asterisks represent peaks of >4N DNA content, present at 48 hours but absent at 72 hours after siRNA treatment. (B) Western blot analysis of fractions shown in A after elutriation (time 48 hours). siRNA to HEF1 depletes HEF1 by 75-90% versus Scr control (based on NIH Image analysis of scanned films). Asterisk marks a nonspecific cross-reacting band, which serves as one loading control: additionally, blots were stripped and reprobed with antibody to β-actin.

Reciprocally, the plane and symmetry of mitotic division leads to the segregation of proteins that reinforce and extend polarity signals. AurA has been shown to be required for this latter process. In *C. elegans*, depletion of AurA (*air-1*) causes defective segregation of P-granules and the protein Pie-1, indicating loss of mitotic asymmetry.[117] In Drosophila, flies with mutated AurA are unable to properly segregate the cell fate determinant Numb, with Numb distributed around the cell cortex instead of polarized in one daughter cell.[118] The polarity machinery (Dlg, Pins, Bazooka/Par3, and aPKC) also specifies asymmetric Numb localization; how AurA signals might interact with this machinery is not clear. A possible role for AurA in governing localization of proteins to focal adhesions or adherens junctions in higher eukaryotes has never been investigated to our knowledge, although intriguingly, our data suggest that mutation of the AurA phosphorylation site on HEF1 influences the ability of HEF1 to return to focal adhesions at the end of mitosis (results not shown).

As noted above, centrosomes give rise to basal bodies in non-proliferating cells, and a number of proteins are shared between centrosome, basal body, and cilia (or flagella, in lower eukaryotes). Bolstering the idea of focal adhesion/centrosome cross-signaling, some recent studies have established relationships between Aurora, Cas proteins, Pak and these additional structures. In the algae *Chlamydomonas*, the AurA ortholog (CALK) is essential for the regulation of flagellar disassembly.[119] CALK itself is phosphorylated (presumably affecting its activity) in response to an array of stimuli normally promoting flagellar resorption. The identity of the proteins transmitting resorption signals to CALK is not known.[119] Nephrocystin and polycystins are proteins that are evolutionarily conserved, cilia-associated proteins that are abundant in renal cells in mammals. Mutations in these proteins are associated with a variety of polycystic kidney disorders;[120,121] studies of their orthologs in lower eukaryotes such as *C. elegans* indicate the defects may involve sensing or response of external chemical or physical signals.[121,122] p130Cas has been identified as an interactor for cilia-associated proteins, including nephrocystin[121,122] and polycystin-1,[123] providing functional coupling between the cilium and proteins including FAK, paxillin, and other focal adhesion components.[122,123] HEF1 mRNA is particularly abundant in kidney tissue:[71] specific localization of HEF1 to cilia, and in renal cells, is currently under investigation. An exciting recent study has also implicated specific Pak kinase activity at the cilium in quiescent cells, where it has been proposed to contribute to environmental sensing and tissue homeostasis.[24] The fact that these recent studies have established relationships between Aurora, Cas proteins,

and Pak and these additional centrosome-related structures further buttresses the idea of focal adhesion to centrosome (to cilia?) cross-signaling,

It has long been known that loss of cell attachment induces defective cytokinesis, and arrest in early G_1.[124,125] As described above, AurA hyperactivation or overexpression promotes defective cytokinesis, and influences the activity of p53, with some studies finding that inactivation of the p53 checkpoint is necessary to promote AurA-dependent cell transformation. Like AurA, HEF1 overexpression and depletion induce M phase defects.[3] We have begun to investigate interactions between HEF1 and the post-mitotic checkpoint machinery. As shown in Figure 3, elutriation of populations of p53-positive MCF7 cells with HEF1 depleted for 48 hours initially reveals a significant fraction of cells have >4N DNA content. However, when these cells are collected, replated, and cell cycle compartmentalization reassayed after 24 hours, the majority of the >4N cells are lost. This implies loss of HEF1 is not able to overcome the post-mitotic checkpoint, and places HEF1 on a signaling pathway relevant to detachment-induced cell cycle arrest or apoptosis. Intriguingly, the HEF1-interacting protein FAK has been shown both to localize to the centrosome,[126] and to interact directly with p53.[127] Both HEF1[3,99] and FAK[128] are subject to substantial changes in phosphorylation during mitosis. These phosphorylations influence the ability of these proteins to associate with different partners.

Speculatively, HEF1 and associated proteins such as FAK and Pak may act in part as attachment-sensing checkpoint proteins at mitotic entry and exit. Movement of these proteins from the basal cell surface to the centrosome at G_2/M may provide a signal that cells have successfully disassembled focal adhesions, and are ready for mitotic rounding. Later in M phase, the destruction or phosphorylation of these proteins to remove them from the mitotic machinery, and their reinstatement at focal adhesions at cytokinesis may be a licensing event for cell reattachment and progression through G_1 phase. On the other hand, it is also well-established that Cas proteins and FAK influence G_1 progression by other means, exclusive of dialog with the checkpoint machinery: for example, FAK regulates cyclin D1 expression,[129] as do small GTPases and Cas effectors such as Rac;[130,131] while Cas proteins positively regulate serum response proliferation signals.[89] Separating the various threads connecting AurA and Cas proteins to the control of cell division will take some time.

CONCLUSIONS AND FUTURE QUESTIONS

Characterization of proteins at the intersection of attachment, mitotic, and checkpoint signaling might be expected to offer important insights into cancer development, given that the deregulation of such proteins might simultaneously promote not only metastasis and tumor cell survival, but also genomic instability. Intriguingly, a recent study mutating genes associated with asymmetric cell division in Drosophila neuroblasts demonstrated that loss of Pins, Numb, and others resulted in the creation of tumors with some properties of stem cells, characterized by genome instability and centrosome alterations.[132] In higher eukaryotes, it is difficult to track the genetic and epigenetic changes associated with tumor cell initiation, because by the time tumors have become large enough to detect, additional changes may have occurred. At present, the relationship of the status of AurA and human cancer initiation is complicated, with some studies identifying overexpression of AurA in large tumors, and others showing it as an event in early tumors, subsequently selected against (also see discussion in ref. 133): for AurA and other proteins such as p130Cas, HEF1, and associated factors, more investigation is required.

A fundamental question arising from these many converging studies is the relationship between the cell asymmetry control machinery and the etiology of most human cancers. Suggestively, a significant number of the asymmetry control proteins are almost by definition exclusively or predominantly expressed in polarized cell types, such as epithelial or neuronal cells. This may contribute to the predisposition of such cells (rather than nonpolarized fibroblast or stromal cells) to form solid tumors, based on their possession of an apparatus that connects more vital cell processes. In the past several years, studies of the growth of cultured cells in more natural three-dimensional matrix environments has begun to reveal unexpected convergence between polarization cues and many cancer-related signaling processes, that differ from previous findings made in cells grown by traditional culture in two-dimensions.[134-136] Extension of these studies to include analysis of mitotic processes is likely to tie together the sequence and interdependence of events leading to tumorigenesis.

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The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome

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Although HEF1 has a well-defined role in integrin-dependent attachment signalling at focal adhesions, it relocalizes to the spindle asters at mitosis. We report here that overexpression of HEF1 causes an increase in centrosome numbers and multipolar spindles, resembling defects induced by manipulation of the mitotic regulatory kinase Aurora-A (AurA). We show that HEF1 associates with and controls activation of AurA. We also show that HEF1 depletion causes centrosomal splitting, mono-astral spindles and hyperactivation of Nek2, implying additional action earlier in the cell cycle. These results provide new insight into the role of an adhesion protein in coordination of cell attachment and division.

HEF1 is a member of a group of scaffolding proteins that includes p130Cas and Efs/Sin[1,2]. This group of Cas proteins localize to focal adhesions in interphase cells, and act as intermediates in a variety of integrin-dependent signalling processes, including the establishment of cell attachments, migration and cell survival signalling. In 1998, we proposed that HEF1 might have a previously unsuspected function in mitosis[3], based on the observation that the HEF1 protein relocalized from focal adhesions to the mitotic spindle asters in M phase. Since that time, reports have appeared that suggest that other focal adhesion complex proteins, such as zyxin[4], paxillin[5], FAK and Pyk2 (ref. 6) associate with the mitotic spindle or other relevant structures, such as the microtubule-organizing centre (MTOC) or centrosome. Recent work has emphasized the dual activity of centrosomes in contributing to the control of cell polarization in interphase cell migration[7,8], but also in coordinating assembly of the mitotic spindle in M phase[9]. Centrosomally associated signalling proteins such as the Aurora-A (AurA) kinase also govern the timing of mitotic entry[10], for instance by regulating the activation of cyclin B1 (ref. 11). In this study, we demonstrate a requirement for HEF1 in activation of AurA and Nek2 (ref. 12) — a second kinase important for centrosome cohesion — and we provide additional data indicating that HEF1 provides a novel bridge that coordinates cell attachment and cell division processes in mammalian cells.

RESULTS

Cell-cycle-regulated HEF1 localization

HEF1 localizes to the centrosome of MCF-7 cells in a cell-cycle-regulated manner (Fig. 1a), with the centrosomal signal lowest in G1, and strongest in G2/M cells. This corresponds to relatively low levels of HEF1 detectable in G1 and to the fact that the bulk of HEF1 in interphase cells localizes to focal adhesions[3] (Fig. 1b, c). As HEF1 levels increase during G2, a slower migrating, hyperphosphorylated species becomes more apparent, as we have previously reported[3]. At mitotic entry, a significant fraction of HEF1 moves to the spindle, and HEF1 is no longer detectable at the centrosome at cytokinesis. The endogenous HEF1 localization pattern described here disappeared following HEF1 depletion with siRNA, supporting signal specificity (see Supplementary Information, Fig. S1A, B). Furthermore, a HEF1-specific mouse monoclonal antibody, mAb-14A11, transiently overexpressing GFP-fused HEF1, generated the same pattern (see Supplementary Information, Fig. S1C–E). Finally, the HEF1 signal in the vicinity of the centrosome was coincident with the patterns seen for multiple centrosome-associated proteins, including γ-tubulin, c-Nap-1, pericentrin, ninein and Nek2 (see Supplementary Information, Fig. S1F). Using GFP- and FLAG epitope-fused HEF1 derivatives, we mapped the minimal sequence determinants that are necessary for localization of HEF1 to the centrosome as being HEF1 amino acids 1–405 (Fig. 1d, e). This sequence contains the SH3 domain and SH2-binding site-rich domains[2], with sequences between 363–405 being an essential localization determinant. In addition to this fragment, carboxy-terminal derivatives of HEF1 (aa 351–653 and 654–834) also showed weaker association with the centrosome, suggesting that more than one interaction contributes to the localization, analogous to the discrete focal adhesion targeting elements within HEF1 (ref. 13).

Up- or downregulation of HEF1 expression causes centrosomal and spindle abnormalities

To determine the functional significance of HEF1 association with the centrosome and the mitotic spindle, we established three independent systems for manipulation of HEF1 in MCF-7 cells. First, we overexpressed

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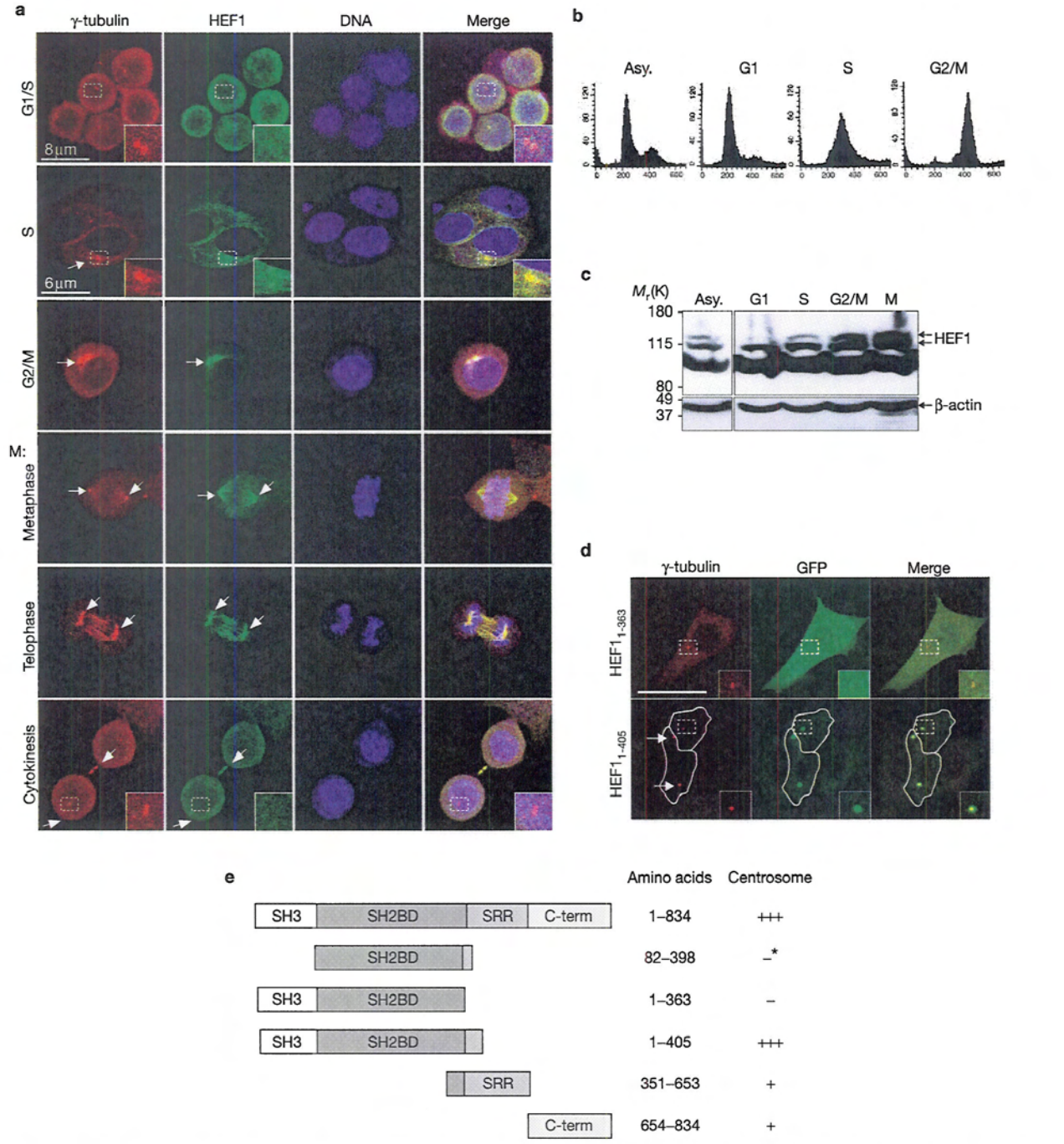

Figure 1 HEF1 localization to the centrosome: cell-cycle and sequence dependence. (**a**) Cell-cycle-synchronized populations were analysed for HEF1 localization to the centrosome and mitotic apparatus. HEF1 is indicated in green, and visualized with anti-HEF1-SB-R1 antibodies, as previously described[3]. γ-tubulin (red) is used to indicate centrosomes (arrows). DNA (blue) becomes condensed at mitotic entry. Enlarged views of boxed centrosomes are shown in the bottom right corners; arrows mark centrosomal location. Images are merged confocal sections. Scale bar of 8 μm applies to top row; 6 μm scale bar applies to all other images. (**b**) FACS analysis demonstrating asynchronous (Asy.) MCF-7 cells, and MCF-7 cells synchronized in G1, S and G2/M phases, as used for immunofluorescence analysis. (**c**) Western analysis of HEF1 levels in the indicated phases of the cell cycle, with β-actin as loading control. The HEF1 doublet represents two phosphorylation-induced isoforms of HEF1 with relative molecular masses of 105,000 (M_r 105K) and 115,000 (M_r 115K) (hyperphosphorylated)[10]. The broad band migrating at ~95K is a non-specific, cross-reacting species detected with the rabbit polyclonal antibody, described in ref. 10 (also see Supplementary Information, Fig. S1A). (**d**) Cells transfected with plasmids expressing GFP–$HEF1_{1-363}$ or GFP–$HEF1_{1-405}$, and stained with antibody to γ-tubulin (red). White lines in lower panels outline cell peripheries. Boxed centrosomes are enlarged in insets; arrows indicate other examples. (**e**) Fragments of HEF1 analysed as GFP- or FLAG epitope-tagged fusion proteins, amino acids and the degree of localization to the centrosome are shown. The degree of centrosomal localization was estimated by measuring the signal intensity at the centrosome in the same set of experiments (data not shown). $HEF1_{1-834}$ is full-length HEF1. Asterisk, protein poorly expressed.

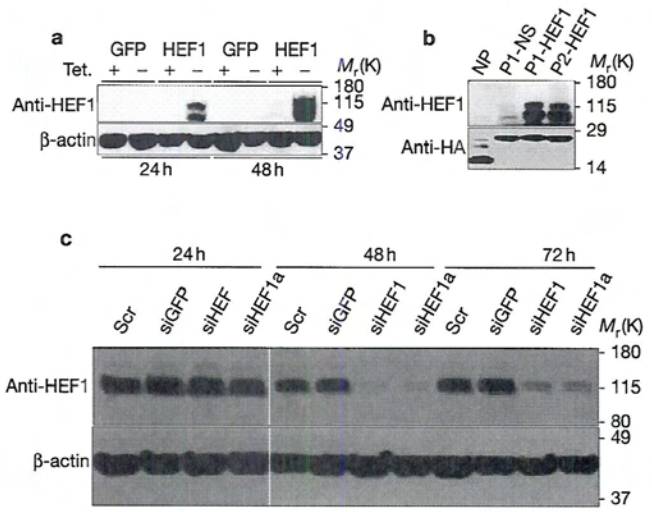

Figure 2 Overexpression, stabilization and depletion of HEF1. (**a**) MCF-7 cells with tetracycline-repressed expression (MCF-7-tTA) of stably integrated HEF1 or GFP in the presence (+) or absence (–) of tetracycline, measured at 24 or 48 h after medium change. Western analysis with antibody to HEF1 demonstrates induction following tetracycline removal. β-actin was used as a loading control. (**b**) Western blot analysis of MCF-7 cells infected by retroviruses expressing HA/thioredoxin-tagged peptide fusion proteins (HA–TRX). Levels of HEF1 are stabilized by specific HA–TRX peptides (P1-HEF1, P2-HEF1), but not by non-specific HA–TRX peptides (P1-NS), or by HA–TRX with no peptide inserted (NP). Antibody to HA shows comparable expression of HA–TRX fusions in all lanes. (**c**) Western blot analysis of MCF-7 cells treated with siRNAs to HEF1 (siHEF1 and siHEF1a) or a scrambled (Scr) or GFP (siGFP) oligonucleotide duplex shows efficient and specific HEF1 depletion at 48 and 72 h time points. The blot was stripped and re-probed with β-actin as a loading control. All lanes shown were run on a single gel; white lines here and in the following figures indicate excision of intervening bands.

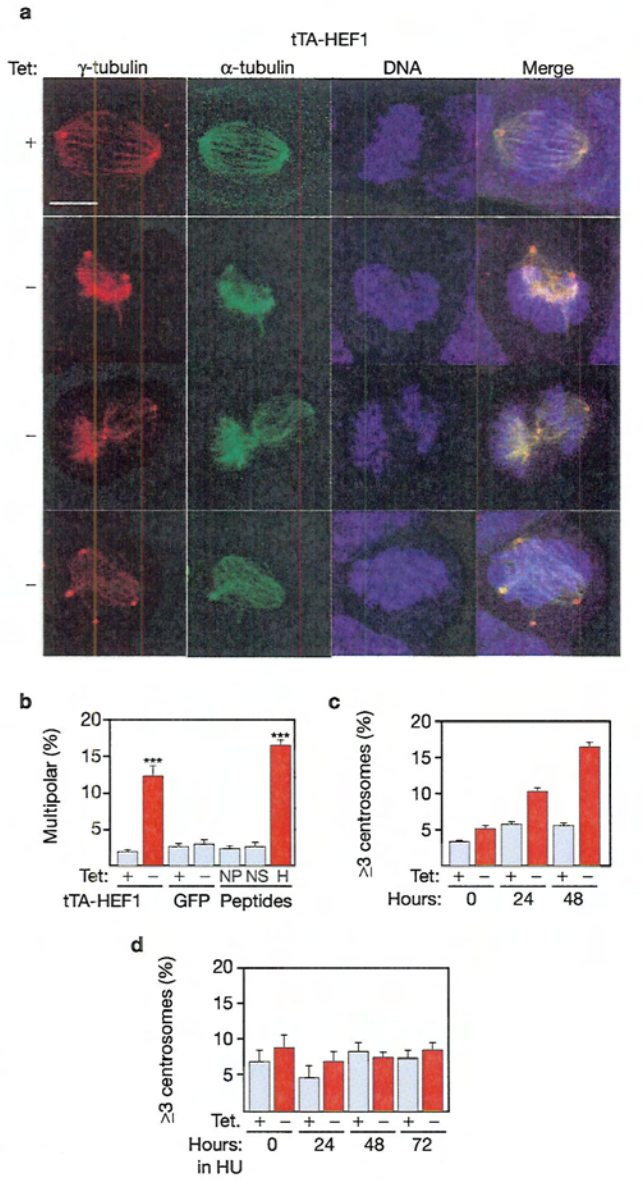

Figure 3 Overexpression and stabilization of HEF1 induce supernumerary centrosomes and multipolar mitotic spindles. (**a**) MCF-7 cells with tetracycline-repressed HEF1 (tTA-HEF1) were un-induced (top row) or induced by tetracycline (Tet) removal (bottom 3 rows). Cells were treated to visualize DNA (blue) α-tubulin (green) and γ-tubulin (red) for immunofluorescence; representative mitoses are shown. Scale bar, 4 μm. (**b**) Quantification of multipolar spindles scored in cells with induced (–Tet) or un-induced (+Tet) HEF1 or GFP expression, or treatment with peptides stabilizing endogenous HEF1: specific for HEF1 (H), non-specific (NS), or peptide negative control (NP) HA–TRX fusions. The histogram indicates the percentage of cells with multipolar spindles under different conditions. Red bars indicate conditions with increased HEF1 levels. Three independent experiments were performed, resulting in the assessment of 150 mitoses in total for each condition. ***$P < 0.001$ versus negative control condition. (**c**) tTA-HEF1 cells were first synchronized using a double thymidine block, then released into medium with tetracycline (blue; HEF1 repressed) or without tetracycline (red; HEF1 induced), and the number of centrosomes scored at 0, 24, or 48 h. The percentage of cells with ≥ 3 centrosomes was recorded. In three experiments 150 cells were counted. ***$P < 0.001$ difference, minus versus plus tetracycline for each time point; difference at 0 h is not significant. (**d**) tTA-HEF1 cells were presynchronized in thymidine, then placed in medium with hydroxyurea (HU), and with or without tetracycline, and centrosomes were scored up to 72 h. In three experiments 150 cells were counted. No significant differences were seen. Error bars indicate standard error of the mean (s.e.m.).

the full-length HEF1 protein under control of a tetracycline-repressible promoter (Fig. 2a). Second, based on previous data indicating HEF1 cleavage and proteolysis at mitosis and apoptosis (discussed further below), we stabilized full-length endogenous HEF1 using peptide aptamers targeted to a previously mapped HEF1 cleavage site[13,14] (Fig. 2b). Third, we used an siRNA approach to deplete endogenous HEF1, using two independent siRNAs (siHEF1 and siHEF1a; Fig. 2c and see Supplementary Information, Fig. S2A). For each manipulation, we used a matching negative control set (corresponding to tetracycline-regulated GFP, for overexpression), non-specific peptides, scrambled siRNA, a GFP-targeted siRNA and, in some cases, a p130Cas-targeted siRNA for depletion (Fig. 2c and see Supplementary Information, Fig. S2A).

Both overexpression of exogenous HEF1 and peptide stabilization of endogenous HEF1 induced a high frequency of cells with spindle defects by 48 h following cell treatment (Fig. 3a, b and Supplementary Fig. S3A, B). Most notable was the increase in the number of cells with multipolar spindles, which represents 12% of the mitotic cell population with overexpression, and >16% with specific HEF1-targeted peptides, as compared with 2–3% for all negative controls. For every cell with multipolar spindles, each spindle originated from a γ-tubulin-, pericentrin- or GFP–centrin-positive structure (Fig. 3a; see Supplementary Information, Fig. S3A; and data not shown). In other cells, whereas no multipolar spindles were observed, the spindle was nevertheless defective. A common phenotype was the presence of 'bent' spindles (Fig. 3a), which would arise if the spindle poles had not fully moved to opposite sides of the cell. Overexpression of HEF1, or introduction of HEF1-stabilizing peptides,

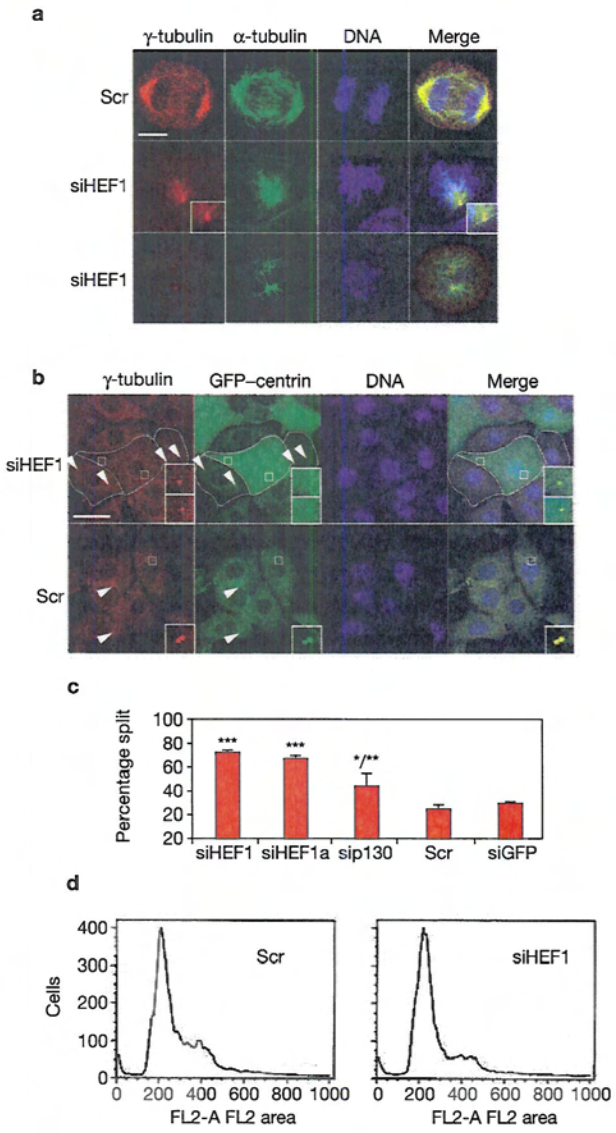

Figure 4 Depletion of HEF1 induces centrosomal splitting and a mono-astral mitotic spindle. (**a**) MCF-7 cells were transfected with either a control scrambled (Scr; top panels) siRNA, or an siRNA specific for HEF1 (siHEF1; middle and bottom panels) for 48 h, then processed for immunofluorescence using markers to γ-tubulin (red), α-tubulin (green) and DNA (blue). Shown are representative mitoses. Insets show enlarged views. Scale bar, 5 μm. (**b**) Immunofluorescence analysis of MCF-7 cells with stably expressed GFP–centrin2 (green) 48 h post-transfection of scrambled (Scr) or HEF1-directed (siHEF1) siRNAs was used to calculate the frequency of split (top panels) versus closely paired (bottom panels; Scr) centrioles (indicated by arrows, with boxed examples shown in insets). The centrioles were considered as split if the distance between them was 2 μm or more[12]. Results shown are for depletion with siHEF1; comparable phenotypes are seen with siHEF1a. DNA is shown in blue. Scale bar, 5 μm. (**c**) For quantification, >150 cells were counted from each of at least three separate experiments for HEF1-depleted (siHEF1) or control (Scr) siRNA-treated cells, and two experiments for sip130 and siGFP. All counting was done at a time point after 48 h of siRNA treatment. ***$P < 0.001$ for siHEF1 versus either Scr or siGFP; */**$P < 0.01$ versus Scr and $P < 0.05$ versus siGFP. Error bars indicate s.e.m. (**d**) FACS analysis of cells depleted with indicated siRNAs at 48 h post-transfection.

was also found to consistently induce supernumerary centrosomes, with >10% of all cells containing in excess of three centrosomes. (Fig. 3a, c and see Supplementary Information, Fig. S3A, B). Abnormally increased numbers of centrosomes can arise from either deregulation of the centrosomal duplication cycle during S phase, or as a result of failed cytokinesis[15]. We found that the increased numbers of centrosomes accumulated gradually over 24–48 h following HEF1 induction (Fig. 3c), and were due to secondary defects in cytokinesis, because centrosomes did not accumulate in HEF1-overexpressing cells held in hydroxyurea (Fig. 3d).

In contrast to the results with overexpressed HEF1, depletion of HEF1 induced a high percentage of cells with mono-astral spindles, containing two γ-tubulin-positive structures (Fig. 4a; centre row), or with poorly formed spindles (Fig. 4a; bottom row). The centrosomes of cells with defective spindles showed weaker reactivity with antibody to γ-tubulin than did cells with undepleted HEF1 (Fig. 4a). Furthermore, the spindles were consistently less reactive with α-tubulin antibody, particularly in cells with less well-formed spindles (see Supplementary Information, Figs S1B and S2B). Parallel staining that was done with antibody to HEF1 indicated that HEF1 itself was effectively depleted in individual cells with noticeable phenotypes (see Supplementary Information, Fig. S2B, bottom row); the most pronounced phenotypes were observed in cells with the greatest degree of HEF1 depletion. HEF1-depleted non-mitotic cells also manifested centrosomal abnormalities. Two distinct, widely separated, GFP–centrin-positive structures (Fig. 4b and see Supplementary Information, Fig. S2B) were observed in >70% of HEF1-depleted cells but in 25–30% of cells treated with a scrambled siRNA (Fig. 4c). Normally, two widely separated centrosomes are not observed until the G2/M transition[15,16]. This increase in the frequency of split centrosomes was observed with both HEF1-directed siRNAs, but with neither of the two non-specific siRNAs; a weaker effect was seen with a p130Cas-directed siRNA (Fig. 4c). Fluorescence-activated cell sorting (FACS) analysis of HEF1-depleted cells versus scrambled siRNA-treated controls confirmed that their cell-cycle profile does not show G2 enrichment (Fig. 4d). Hence, the primary defect with HEF1 depletion is probably one of centrosome cohesion resulting in premature splitting, rather than a secondary consequence of altered cell-cycle compartmentalization.

HEF1 interacts with AurA and is required for the activation of AurA kinase

The phenotypes described above for HEF1 depletion and overexpression are similar to those reported for inhibition or overexpression of other proteins known to regulate centrosomal maturation and cell-cycle progression, including the AurA kinase[17,18]. Indeed, whereas in HEF1-depleted MCF-7 cells in the G2 phase of the cell cycle total levels of AurA at the centrosome were similar to those found in MCF-7 cells treated with a matched scrambled siRNA (Fig. 5a, b), levels of phospho-AurA (T288, indicative of kinase activation[11]) were, in contrast, greatly reduced or absent under conditions of HEF1 depletion (Fig. 5a, b). This implied that HEF1 has an important role in the activation of AurA. This role could be direct, with HEF1 being a physical component of an AurA activation complex, or it might be indirect, with HEF1 causing defects at an early stage of the centrosomal cycle that interfere with AurA activation. AurA and HEF1 co-precipitated from whole-cell lysates, with the greatest levels of association being in cells in G2 (Fig. 5c). This suggests that HEF1 control of AurA activation might be direct, through a physical interaction with AurA itself or with an AurA-containing complex.

Using a series of GST-fused truncations of HEF1, we determined that GST–$HEF1_{1-363}$ was the minimal sequence required to efficiently pull down AurA *in vitro* (Fig. 6a). Our earlier results (Fig. 5a, b) predicted that HEF1 interaction with AurA might help to activate the kinase.

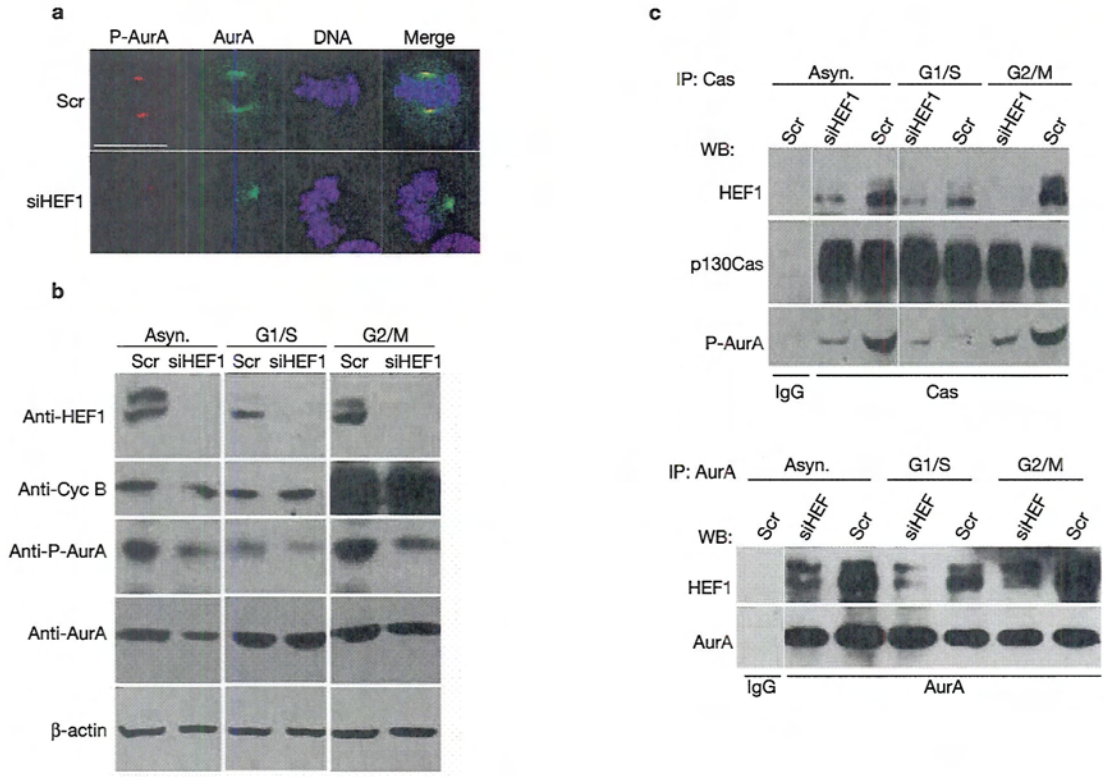

Figure 5 HEF1 associates with AurA and controls AurA activation. (**a**) MCF-7 cells were depleted with HEF1-directed siRNA (siHEF1) or a scrambled control (Scr). Mitotic cells stained with antibodies directed at AurA (green), phospho-AurA/T288 (P-AurA; red) and DNA (blue) are shown. Scale bar (5 μm) applies to all panels. (**b**) MCF-7 cells were transfected with scrambled control (Scr) or HEF1-directed (siHEF1) siRNA for 48 h, then collected and part of the sample elutriated. Non-elutriated (Asyn.), G1/S, or G2/M enriched populations were used for western blot analysis using the antibodies indicated (left). White vertical lines indicate where intervening (irrelevant) lanes run on the gel have been excised from the image. (**c**) MCF-7 cells prepared as in **b** were used for co-immunoprecipitation. Top: immunoprecipitation with control IgG or Cas antibody (cross-reactive with p130Cas and HEF1; efficient direct precipitation of both proteins), and western blot using antibodies as indicated on the left. Bottom: immunoprecipitation with control IgG or AurA antibody, and western blot as indicated. For each experiment, co-immunoprecipitation is compared in cells depleted for HEF1, or treated with scrambled siRNA, to confirm the specific requirement of HEF1 in co-immunoprecipitation.

Full-length HEF1 is not stable as a recombinant purified protein, prohibiting a direct *in vitro* test for this idea with the native protein (data not shown). However, as an alternative approach, we titrated the GST–$HEF1_{1-363}$ minimal AurA-interacting domain versus GST into an *in vitro* kinase reaction containing recombinant AurA purified from bacteria (Fig. 6b). Increasing levels of GST–$HEF1_{1-363}$, but not of GST, clearly induced the autophosphorylation of AurA and the ability of AurA to phosphorylate a histone H3 substrate, indicating that the association with HEF1 is sufficient to promote AurA catalytic activity[11]. Indeed, a higher level of activated AurA was observed in cells overexpressing HEF1, in contrast to the lower levels of AurA activation seen with HEF1 depletion (Fig. 6c). Furthermore, we found that both GST–$HEF1_{1-363}$ and $HEF1_{1-405}$ were phosphorylated by recombinant activated AurA *in vitro* (Fig. 6d).

Although the AurA consensus site remains poorly defined, an RHQSer 296LSP motif closely resembles a site phosphorylated by the Aurora yeast orthologue Ipl1p (ref. 19). We used mass spectrometry analysis of *in vitro* phosphorylated GST–$HEF1_{1-363}$ to confirm *in vitro* phosphorylation of this site (data not shown), and mutated Ser 296 into alanine (unphosphorylatable) or glutamic acid (mimicking constitutive phosphorylated HEF1) alone or together with an adjacent serine, Ser 298. All Ser 296 mutants were no longer phosphorylated by AurA (Fig. 6e). However, whereas alanine mutants of GST–$HEF1_{1-363}$ maintained the ability to interact with AurA and activate the kinase, glutamic acid mutants of HEF1 lost both abilities (Fig. 6f). In parallel, we compared the relative interaction of HEF1 with AurA in the presence or absence of ATP *in vitro* (see Supplementary Information, Fig. S4A). We found that AurA co-immunoprecipitated much more efficiently with unphosphorylated HEF1, supporting the results obtained with the mutants.

In vivo, we then compared the ability of GFP-fused HEF1, $HEF1^{S296E}$, $HEF1^{S296E/S298E}$ and $HEF1^{S296A/S298A}$ to immunoprecipitate AurA (Fig. 6g). Whereas $HEF1^{S296A/S298A}$ was similar to HEF1 in interacting with AurA, both phosphomimic variants were severely impaired for AurA interaction, similar to the *in vitro* results. Together, these data suggest a model in which an initial interaction of HEF1 with AurA prior to mitotic entry activates AurA, which then phosphorylates HEF1, promoting dissociation of the two proteins. Amino acids 1–405 are a minimum determinant of strong HEF1 association with the centrosome *in vivo* (Fig. 1d, e), with a critical localization determinant located in the serine-rich amino acids from 363–405. We transfected GFP-fused truncation derivatives of HEF1 into the MCF-7 cells, immunoprecipitated with antibody to GFP, and confirmed that GFP–$HEF1_{1-405}$ co-immunoprecipitated with AurA from whole-cell lysates, whereas GFP–$HEF1_{1-363}$ did not (see Supplementary Information, Fig. S4B). We next compared the activation of AurA that had been immunoprecipitated from cells expressing HEF1, $HEF1_{1-363}$ and $HEF1_{1-405}$ (see Supplementary Information, Fig. S4C). GFP–$HEF1_{1-405}$ was like GFP–HEF1 in that it promoted increased activity of AurA against a histone H3

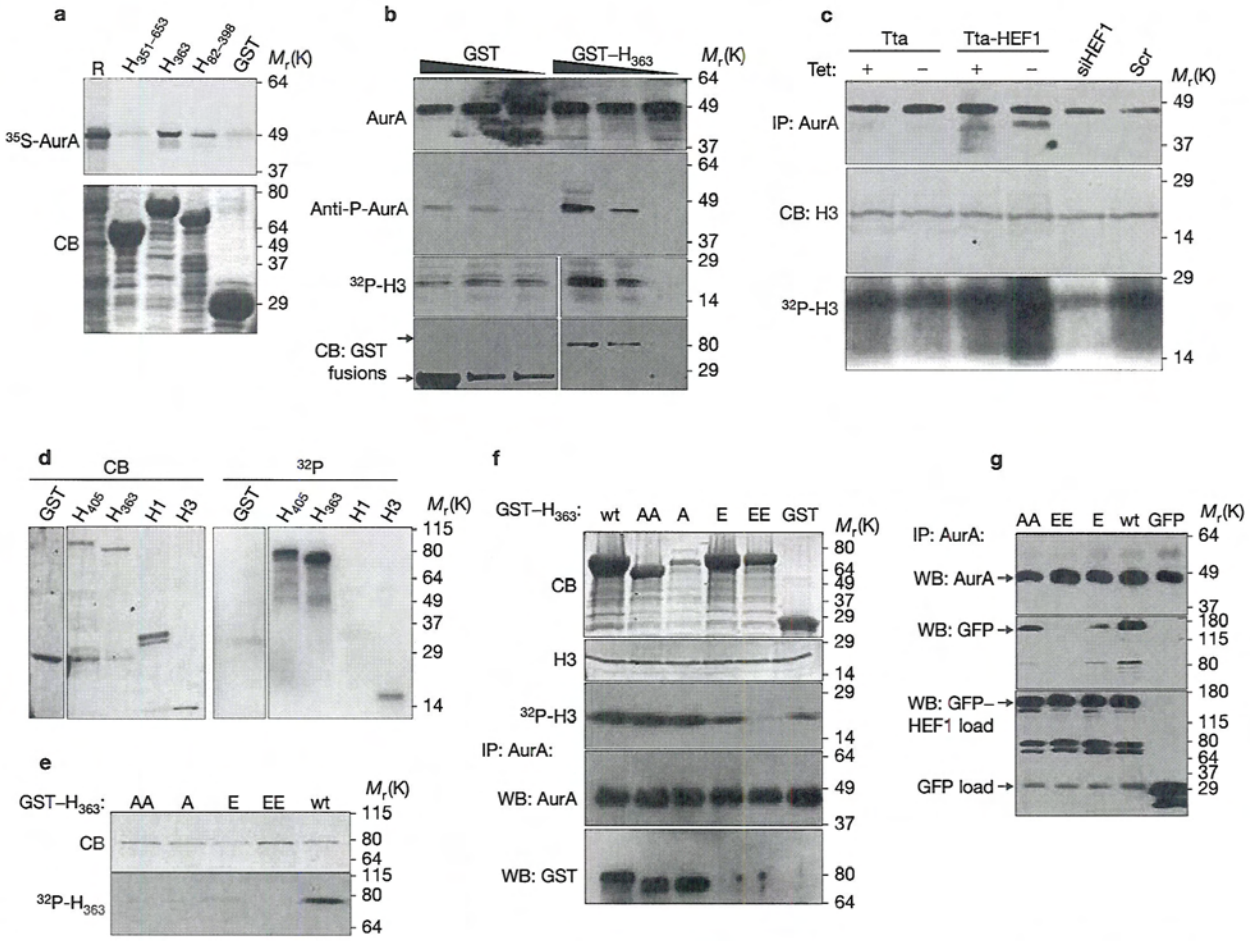

Figure 6 Delineation of the HEF1-AurA interaction. (**a**) *In vitro* translated ^{35}S-labelled AurA was used for pulldowns with GST-fused fragments of HEF1 or GST. Autoradiography shows AurA (top panel), and Coomassie blue (CB) shows GST fusions (bottom panel). In lane 'R', 20% of a total AurA ^{35}S-labelled reaction mixture is shown; for each pulldown, a complete reaction mixture is used. (**b**) Decreasing quantities (4, 2, or 1 μg) of GST-fused $HEF1_{1-363}$ or GST were used in an *in vitro* kinase reaction with recombinant AurA. Reactions were visualized with antibody to AurA or phospho-AurA/T288, or with Coomassie blue to show GST fusions. Phosphorylated histone H3 was visualized by autoradiography. (**c**) AurA was immunoprecipitated from MCF-7 cells that were tetracycline repressible for vector (tTA) or HEF1 (tTA-HEF1) expression, + or – tet; and additionally, from cells treated with siRNA to HEF1 (siHEF1) or to scrambled control (Scr). The immunoprecipitated AurA was incubated with recombinant histone H3 with γ-^{32}P-ATP in an *in vitro* kinase assay, resolved by SDS–PAGE, and visualized by antibody to AurA, Coomassie blue (CB) staining to detect H3, and autoradiography. (**d**) GST, GST-fusion proteins, or histone H1 or H3 (CB; left) were incubated with AurA and γ-ATP-^{32}P *in vitro*. Right: autoradiograph of phosphorylated proteins. (**e**) GST–$HEF1_{1-363}$ wild type (wt), or with alanine or glutamic acid mutations (S296A (A), S296A/S298A (AA), S296E (E), S296E/S298E (EE)) were incubated with AurA and γ-ATP-^{32}P *in vitro*. Top: CB shows GST–HEF1 fusions. Bottom: autoradiograph of phosphorylated GST-fusions. (**f**) The GST-fused derivatives of HEF1 described in **e** were mixed with recombinant AurA and histone H3 (H3) in the presence of γ-ATP-^{32}P, incubated, and the level of histone-H3 phosphorylation was determined by autoradiography. Top, CB shows GST fusions; second row, input histone H3; third row, phospho-histone H3. After incubation, immunoprecipitated AurA (IP:AurA) was probed with antibody to AurA and GST. (**g**) GFP-fused full-length HEF1 (wt or with mutations described in **e**) were transfected into MCF-7 cells. After 24 h, sequential western blot analysis of immunoprecipitated AurA was done using anti-AurA and anti-GFP (top and middle panels). Bottom: anti-GFP shows expression of GFP fusions in total lysate; bands at M_r ~ 70–80K are degradation products.

substrate, whereas $GFP–HEF1_{1-363}$ did not. Together, these results imply that a primary role of aa 363–405 in promoting the HEF1–AurA interaction *in vivo* is through localizing HEF1 to the centrosome, where endogenous AurA is concentrated (Fig. 5a). We were unable to test whether $GFP–HEF1_{1-405}$ was similar to GFP–HEF1 in inducing multipolar spindles, because in a survey of hundreds of cells, no mitotic cells overexpressing $GFP–HEF1_{1-405}$ were ever observed, suggesting that this truncation may be disrupting HEF1-dependent processes prior to mitotic entry.

HEF1 negatively regulates Nek2 and contributes to accumulation of pericentriolar material (PCM)

In normal cells, after telophase, centrosomes mature through the cell cycle, accumulating PCM that includes signalling proteins that govern centrosomal duplication and other functions such as microtubule nucleation[15]. Cohesion of the centrosomes is maintained by c-Nap-1: levels of c-Nap-1 are reduced 10-fold at the G2/M boundary, with phosphorylation by the Nek2 kinase, and potentially AurA, Cdk1 and Plk1 promoting its removal from the PCM and centrosomal disjunction, allowing formation of a bipolar mitotic spindle[12,20,21]. Moreover, overexpression of Nek2 induces precocious centrosomal disjunction in interphase[12], whereas AurA depletion does not (see Supplementary Information, Fig. S3C–E). We examined the status of Nek2 and c-Nap-1 (Fig. 7a), and the centrosomal maturation marker ninein (see Supplementary Information, Fig. S4D) at the centrosome in MCF-7/GFP–centrin cells depleted of HEF1. HEF1 depletion reduced the signal intensity of all of these proteins at the centrosome, suggesting a contribution of HEF1 to the stable

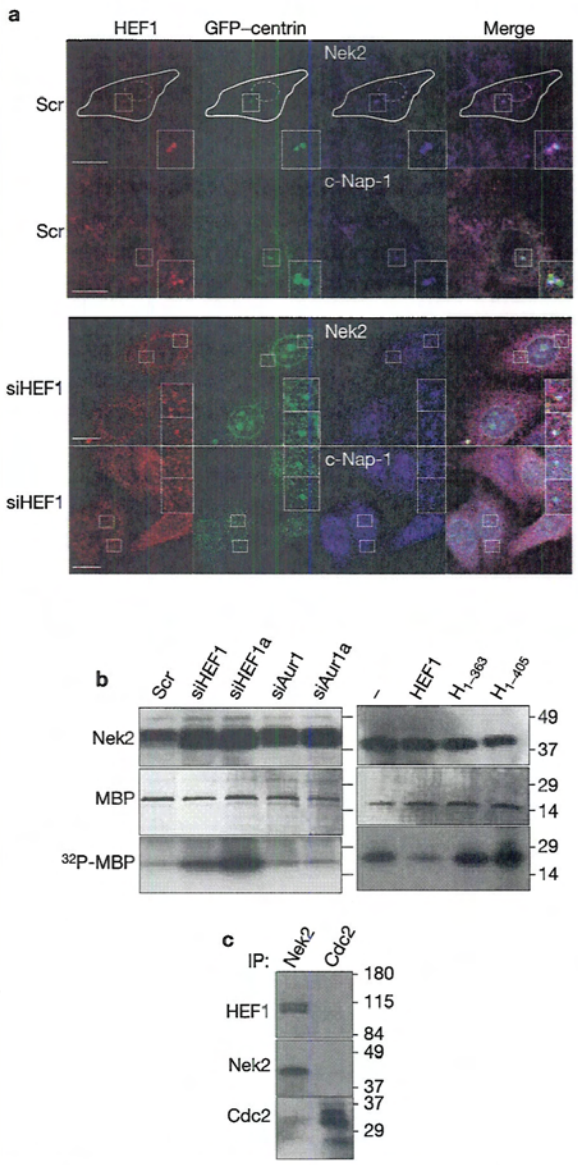

Figure 7 HEF1 depletion affects Nek2 activation and association of proteins with the PCM. (**a**) MCF-7 cells with integrated GFP–centrin were treated with scrambled control siRNA (Scr), or siRNA to HEF1 (siHEF1), and stained for immunofluorescence with antibodies to HEF1, c-Nap-1 or Nek2, as indicated. Scale bars, 10 μm. (**b**) MCF-7 cells were either treated with siRNAs (left panel), or infected with retrovirus expressing full-length HEF1 or HEF1 truncations, as for Supplementary Information, Fig. S4C. Nek2 kinase was immunoprecipitated and used for *in vitro* kinase assays with myelin basic protein (MBP) as a substrate. A comparable level of HEF1 or AurA protein depletion (see Supplementary Information, Fig. S3C), and similar levels of overexpression of HEF1 and truncations (data not shown) was confirmed with antibodies to GFP and/or HEF1and AurA, in whole-cell lysates. (**c**) Antibody to Nek2 or to Cdc2 was used for immunoprecipitation from asynchronous MCF-7 cells. Immunoprecipitates were probed with antibodies to HEF1, Nek2, or Cdc2, as indicated.

assembly of proteins with the PCM. We next asked whether HEF1 regulates Nek2 activation. Nek2 immunoprecipitated from HEF1-depleted cells was much more active in phosphorylating an MBP substrate than Nek2 from control-depleted cells (Fig. 7b). Moreover, overexpression of GFP–HEF1 decreased, whereas overexpression of GFP–$HEF1_{1-405}$ increased activation of Nek2 relative to background levels in cells expressing GFP. As in the other assays, GFP–$HEF1_{1-363}$ exhibited no activity. Finally, we found that endogenous HEF1 and Nek2 co-immunoprecipitated efficiently and specifically from MCF-7 cells (Fig. 7c). Together, these data imply that HEF1 contributes to Nek2 inhibition during the normal cell cycle.

Separation of HEF1 actions at the centrosome and in attachment

The main functions previously ascribed to HEF1 and the Cas family of proteins have been involved in regulation of cell attachment and motility[1,2]. We had previously shown that overexpression of HEF1 induced cell spreading[13,22], whereas overexpression of the HEF1 C terminus had a dominant-negative function on cell attachment, causing cell rounding[13]. To evaluate whether HEF1 control of cell attachment, and regulation of centrosomal splitting, were linked or separable, GFP-fused HEF1 and HEF1 truncations were transfected into MCF-7 and HeLa cells, and the ability of different HEF1 domains to act as dominant negatives by inducing centrosomal splitting versus inhibiting cell attachment was scored (Fig. 8a–c). On the basis of this analysis, full-length HEF1 weakly induced centrosomal splitting but induced spreading. However, the $HEF1_{1-405}$ domain proved to have a potent phenotype, and the $HEF1_{351-653}$ fragment a weaker phenotype, in inducing centrosomal splitting (Fig. 8a), whereas neither affected the degree of cell spreading (Fig. 8b). Conversely, $HEF1_{654-834}$ induced significant cell rounding (Fig. 8b), as reported before, but did not affect centrosomal splitting (Fig. 8a). These results indicate that separable HEF1 domains were required for centrosomal and attachment activities.

In a further test, we determined the consequences of varying the degree of cellular attachment induced by extrinsic stimuli on HEF1-associated centrosomal phenotypes. Cells with induced overexpressed full-length HEF1 (Fig. 8d), depleted HEF1 (Fig. 8e), or overexpressed dominant-negative $HEF1_{1-405}$ (Fig. 8f) were plated on either normal tissue substrates, or on poly-L-lysine, fibronectin, or laminin to increase spreading. Cells plated on the last three substrates were significantly more spread, and were marked by more pronounced paxillin staining at focal adhesion structures (data not shown). However, in scoring the number of supernumerary centrosomes induced by overexpressed HEF1 (Fig. 8d), or the number of split centrosomes induced by removal or dominant-negative blockade of HEF1 function (Fig. 8e, f), the greater attachment status did not affect the observed phenotypes. Together with the earlier results, these findings demonstrate that HEF1-induced cell spreading and enhancement of focal adhesions do not cause the centrosome abnormalities that we have described here; rather, a distinct function of HEF1 is involved.

DISCUSSION

The results of this study for the first time establish the focal adhesion protein HEF1 as a regulator of AurA and Nek2 activation, and of centrosome cohesion and amplification. Proteins that were initially defined as components of the cell attachment machinery, including APC[23] and Ajuba[11], have recently been found to also function in cell-cycle controls, and elegant genetic studies in lower eukaryotic models for development such as *Caenorhabditis elegans* (reviewed in refs 24, 25) have begun to allow a model to be elucidated, in which dynamic interconnections between the centrosome and structures at the cell cortex control the plane of mitotic spindle orientation, and cleavage furrow formation. It is likely that this mechanism will prove to be important for higher eukaryotes as well, given the need of many cells to limit cell division to specific planes (for example, to maintain barrier function). An economical view of cellular function would suggest that the re-use of proteins that govern cell

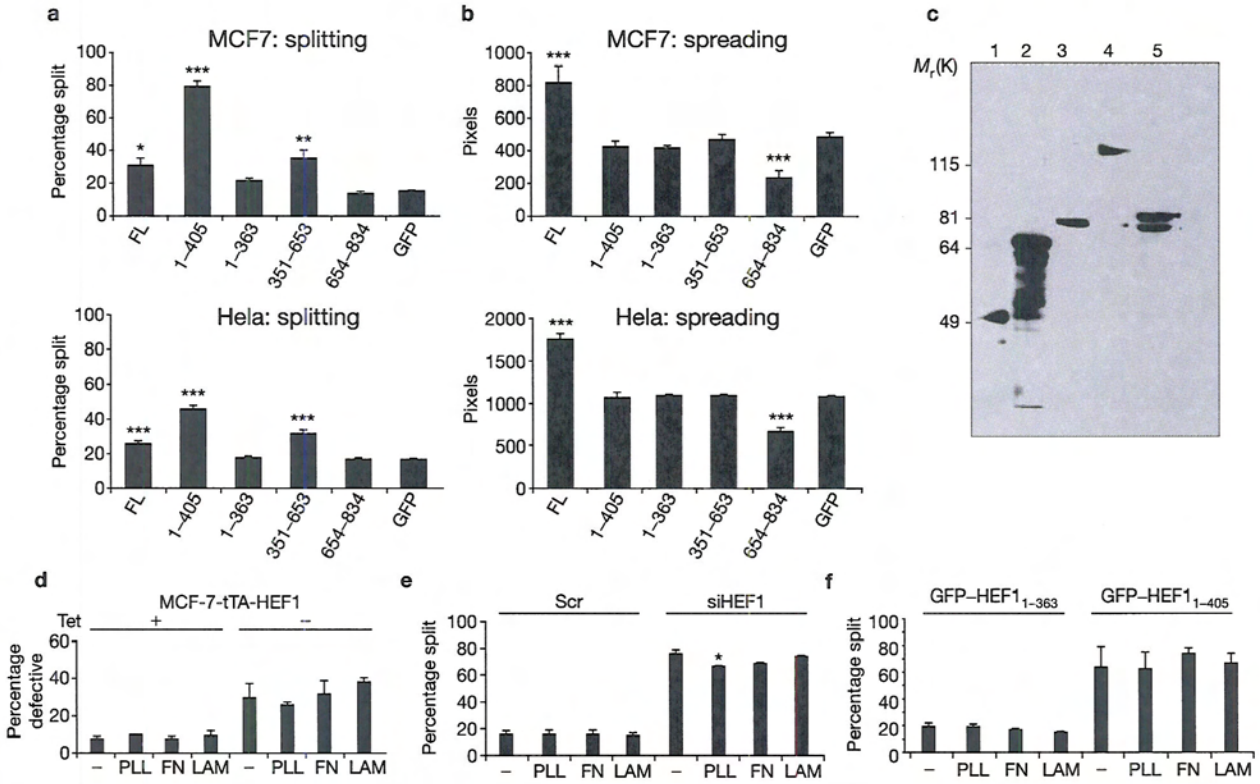

Figure 8 HEF1 activities at the centrosome and in cell spreading. For histograms, $***P < 0.001$; $**P < 0.01$; and $*P < 0.05$, in reference to the GFP control. Error bars indicate s.e.m. (**a**) Centrosomal splitting in GFP-positive cells transfected with HEF1 truncations. Top, MCF-7 cells; bottom, HeLa cells. Bars represent the percentage of split centrosomes. Three (MCF-7) and two (Hela) independent experiments were performed ($n = 100$ cells per experiment). FL, full-length HEF1. (**b**) Cell spreading in GFP-positive cells transfected with GFP-fused HEF1 truncations. Top, MCF-7 cells; bottom, HeLa cells. One hundred cells were counted in each of three experiments. Cell spreading analysis was performed as described previously[13], with calculation of area based on pixels within a traced cell perimeter. Immunofluorescence with α-paxillin was used to confirm increased size and formation of focal adhesions in cells plated on laminin and fibronectin. (**c**) Western analysis of expression of different HEF1 truncations expressed as GFP-fused proteins in MCF-7 cells. Blot was probed with antibody to GFP. Lanes are GFP fusions to: 1, 654–834; 2, 351–653; 3, 1–363; 4, FL; 5, 1–405. (**d**) Mitotic spindle defects induced by HEF1 overexpression in cells plated on different matrices. Tet-repressible MCF-7-derived cells were grown in the presence (+) or absence (–) of tet 48 h after plating on glass coverslips (-), or coverslips coated with poly-L-lysine (PLL), fibronectin (FN), or laminin (LAM). Bars represent the percentage of multipolar or malformed spindles. One hundred mitotic cells were counted in each of three independent experiments. (**e**, **f**) Centrosomal splitting induced by depletion of HEF1 (siHEF1), or overexpression of dominant-negative HEF1 (GFP-fused HEF1$_{1-405}$), in cells plated on different matrices. **e**, MCF-7 cells with integrated GFP–centrin2 were transfected with siRNA containing scrambled sequence (Scr) or targeted to HEF1 (siHEF1). **f**, MCF-7 cells were transfected with GFP-fused HEF1$_{1-363}$ or HEF1$_{1-405}$, and centrosomes were visualized by antibody to γ-tubulin. Following treatment, cells were plated on glass coverslips (-), or coverslips coated with poly-L-lysine (PLL), fibronectin (FN), or laminin (LAM). Bars represent the percentage of split centrosomes. One hundred cells were counted in each of three experiments. For **d**–**f**, the differences induced by matrix were not statistically significant, except $*P < 0.05$ for PLL in **d**.

attachment and cytoskeletal dynamics in interphase cells might not only be efficient, but might also provide a means of synchronizing changes in cell contacts during the mitotic process. It is possible that the pools of HEF1 used for centrosome, mitotic spindle and focal adhesions are completely distinct. However, it may be that migration of proteins such as HEF1 between these structures provides polarity and attachment cues that influence the entry to, and exit from, mitosis. Given the particular abundance of HEF1 in polarized epithelial and lymphoid cell populations, our work would define it as an excellent candidate for such a role.

Our data suggest a model in which in normal cells, HEF1 initially interacts with AurA in G2 prior to AurA activation, with the centrosome being one important point of interaction. In this model, as mitosis approaches, focal adhesion disassembly releases more HEF1, and the increasing interaction of HEF1 with AurA promotes AurA activation. In turn, phosphorylation of HEF1 by the activated AurA reduces the affinity of interaction between the two proteins, perhaps contributing to the relocation of HEF1 away from the centrosome, or perhaps contributing to the preferred interaction of AurA with other partner proteins in the context of the centrosome. AurA activity is known to be regulated by several other protein partners, including TPX2, Ajuba and PP2. In cells depleted of HEF1, AurA does not become activated, suggesting that the association with HEF1 is functionally important. In cells that have HEF1 overexpressed, and are able to associate with the centrosome, the stoichiometry of HEF1 is significantly increased, allowing the protein to continue to interact with AurA in spite of phosphorylation by AurA, and thus promoting elevated AurA activity. For both AurA and HEF1, the centrosomal amplification and multipolar spindles seen with overexpression of the proteins is a secondary consequence of cytokinetic failure. The exact mechanism is yet to be defined, but may involve regulation of a common effector by these proteins.

HEF1-depleted cells have abnormally split centrosomes, which accumulate reduced levels of γ-tubulin in G2 (refs 15, 16, 26), have abnormally reduced accumulation of ninein, c-Nap-1 and other proteins, and are deficient in organizing microtubules at mitosis. These defects are likely to be independent of HEF1–AurA signalling, because AurA depletion does not result in centrosomal splitting (see Supplementary Information, Fig. S3C–E), and may be direct (at the centrosome) or indirect. Our data suggest that HEF1 may normally act to restrain the activity of Nek2 (refs 12, 20), because HEF1 co-immunoprecipitates with Nek2. Nek2 is hyperactivated in cells with depleted HEF1 or dominant-negative $HEF1_{1-405}$ (Fig. 7b, c), with increased Nek2 activation previously reported as being sufficient to induce splitting. HEF1 may have additional activities required for centrosomal cohesion, as hyperactivation of Nek2 is not sufficient to completely remove c-Nap-1 from centrosomes in interphase cells[12], whereas accumulation of ninein is an early step in the maturation of daughter centrosomes to mother centrosomes, and has not been described as being influenced by Nek2. Such a role for HEF1 is separable from any secondary effect due to defects in cell attachment, as different domains of HEF1 caused splitting versus cell rounding. Intriguingly, recent protein interaction studies of the ancestral HEF1/p130Cas homologue in *Drosophila*[27] have suggested that this protein associates with a component of the γ-tubulin ring complex (γ-TuRC), which is important for microtubule nucleation[28]. It is also interesting that we have previously found that the cleavage of HEF1 at amino acid 363 by caspases produces a p55 species[3,13]. Although we originally found this p55 species in both mitotic and apoptotic cells, our ongoing work has suggested that the initial suggestion of HEF1 cleavage at mitosis may have arisen at least in part from contamination of drug-synchronized mitotic populations with apoptotic cells (data not shown). However, the fact that $HEF1_{1-353}$ does not associate with centrosomes or with AurA in cells, whereas the slightly larger $HEF1_{1-405}$ does, implies that cleavage of HEF1 at this site may have a functional significance in disrupting centrosomal function during cell death.

Defining HEF1 as a component of the AurA activation machinery is an important finding, providing evidence of a new channel for cross-signalling between cell adhesion and mitosis. Furthermore, AurA and Nek2 overexpression and hyperactivation have been observed in many tumours, and are associated with genomic instability[29–31]. It was shown for Cas proteins[32] that upregulation of p130Cas and/or HEF1 correlates with poor prognosis in breast cancer. It has additionally been shown by us and others that upregulation of Cas proteins influences the transcription of a number of gene pathways associated with cancer development, enhancing activation of the MAPK pathway and promoting matrix metalloproteinase production (refs 22, 33 and others). It is entirely possible that altered Cas protein levels also induce transcriptional changes that influence genomic stability. Our data imply that beyond their well-defined functions in regulating susceptibility to apoptosis and cell migration, HEF1 and potentially other members of the Cas family may make additional contributions to the processes of cell transformation through the regulation of mitosis. □

METHODS

Plasmids and constructs. FLAG-, GFP- and GST-fused HEF1 and derivatives were expressed from the vectors pCatch-FLAG, pEGFP-C4 (ref. 13) and pGST, respectively. AurA expressed from pCMV-SPORT6-C6 (OpenBiosystems, Huntsville, AL) was used for *in vitro* translation. AurA in pFAST-HT was used for production in baculovirus, and purified by Ni-Sepharose 6FF (Amersham, Piscataway, NJ). HEF1-specific and non-specific peptides[14] were expressed from the retroviral vector pUP. Tet-repressible HEF1 in MCF-7 was made using HEF1 in the expression vector pUST-4. Lentiviral constructs were obtained by cloning GFP–HEF1, $-HEF1_{1-363}$, or $-HEF1_{1-405}$ into pLV-CMV-H4-puro-vector. HEF1 mutants S296A, S296E, S296A/S298A, and S296E/S298E were made using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primer sequences are available on request.

Cell culture. MCF-7 (breast adenocarcinoma) and HeLa (cervical carcinoma) cell lines were grown in DMEM plus 10% fetal calf serum. The MCF-7-GFP-centrin2 and HeLa-GFP-centrin2 stable cell lines were obtained by transfection of parental cells with pEGFP-centrin2 plasmid[34], and G418 selection. Tetracycline-regulated MCF-7-tTa-HEF1 stable cell lines were obtained by first infecting the parental MCF-7-tTa cell line (BD Biosciences, San Jose, CA) with the pUST-4-HEF1 retroviral vector, then selecting with G418/puromycin to produce a mass culture. For analysis of centrosome number in cells that had not undergone mitosis, MCF-tTa-HEF1 cells were plated for 24–72 h +/- tet, with 1 mM hydroxyurea (Sigma, St Louis, MO). Alternatively, after using a double thymidine block in the presence of tetracycline, cells were released and grown for 24 and 48 h in fresh media +/- tet. For growth of cells on poly-L-lysine, laminin, or fibronectin, the procedure described in ref. 13 were used to prepare coverslips. Cells for analysis were plated on these versus uncoated (normal) coverslips, grown for 48 h, then centrosomal composition and spreading were scored. Examination of centrosomes in non-adherent cells plated on poly-HEMA[13] was impossible because onset of apoptosis within 24 h precluded reliable analysis (data not shown). For lentiviral infection, pLV constructs were transfected into the packaging cell line 293-T. After 24 h, media was collected, filtered through a 0.45-μm PVDF filter (Millipore, Billerica, MA), and applied to MCF-7 cells with polybrene for 2 days, with fresh viral supernatant added every 12 h. After 48 h, cells were lysed, analysed by western blot analysis, and used for immunoprecipitation kinase reaction.

Protein expression, western blotting and immunoprecipitation. Recombinant proteins were expressed in BL21 (DE3) bacteria, induced with IPTG, and purified using the MicroSpin GST Purification module (Amersham, Piscataway, NJ). Purified recombinant AurA was purchased from Upstate (Charlottesville, VA). For western blotting and immunoprecipitation, mammalian cells were disrupted by M-PER lysis buffer (Pierce, Rockford, IL) or NET2 buffer plus protease inhibitor cocktail, and whole-cell lysates used either directly for SDS-polyacrylamide gel electrophoresis (SDS–PAGE), or for immunoprecipitation. Immunoprecipitation samples were incubated overnight with antibody at 4 °C, subsequently incubated for 2 h with protein A/G-sepharose (Sigma), washed and resolved by SDS–PAGE. Western blotting was done using standard procedures and developed by chemoluminescence using the West-Pico system (Pierce). Transiently transfected or infected cells were analysed for protein expression at 24–96 h h post-transfection with Lipofectamine 2000 for plasmids, or Oligofectamine for siRNA (both from Invitrogen, Carlsbad, CA). Antibodies used included: rabbit polyclonal antibody to HEF1 (ref. 3) at a 1:100 dilution, mouse monoclonal antibody anti-HEF1 14A11 made for this study (1:500), anti-α-tubulin mouse monoclonal antibody (Sigma; 1:10,000), mouse monoclonal antibody anti-γ-tubulin (GTU-88; Sigma; 1:5,000), anti-p130Cas (BD Biosciences; 1:2,000), anti-AurA (BD Biosciences; 1:1,000) for western, anti-AurA rabbit polyclonal (Abcam, Cambridge, MA; ab1287) for immunoprecipitation, anti-Phospho-AurA/T288 (Cell Signaling, Beverly, MA; 1:1,000), anti-HA-antibody (16B12; BabCo, Berkeley, CA; 1:5,000), mouse monoclonal antibody anti-β-actin (AC15; Sigma; 1:10,000), mouse monoclonal antibody anti-cyclin B (GNS-1; BD Biosciences; 1;1000). Rabbit anti-GFP (Abcam ab290) was used for immunoprecipitation, and mouse anti-GFP (JL-8; BD Biosciences; 1:2,000) was used for western blotting. Monoclonal antibody anti-GST (Cell Signaling; 1:2,000) and rabbit anti-Nek2 (Abcam; 1:200) were used for immunoprecipitation. Mouse anti-Nek2 (BD Biosciences; 1:500) and mouse monoclonal antibody anti-cdc2 antibody (Oncogene, Cambridge, MA; 1:1,000) were used for western blot analysis. Secondary anti-mouse and anti-rabbit HPR conjugated antibodies (Amersham) were used at a dilution of 1:10,000 or 1:20,000.

siRNA. RNA oligonucleotides duplexes (sequences on request) were synthesized targeted to HEF1, AurA and to p130Cas, as well as negative controls including scrambled and GFP-directed sequences (Dharmacon, Lafayette, CO; Ambion, Austin, TX). After transfection of siRNAs, degree of depletion of target proteins was determined by western blot.

Cell synchronization. Cells were incubated for 16–18 h with 2 mM thymidine, washed twice in PBS, then either assayed directly (for observation at the G1/S boundary), or returned to fresh medium and allowed to grow for 9–12 h to observe synchronized progression to mitosis. For synchronization at the G2/M boundary, cells were incubated in 1 μM nocodazole for 14 h, collected by shake-off, washed in PBS, then either re-plated in fresh medium on glass coverslips, cultured at 37 °C for up to 90 min, then fixed for immunofluorescence analysis; or lysed for western and immunoprecipitation analysis. As an alternative drug-free synchronization approach, an elutriating centrifuge (Beckman J, Fullerton. CA) was used to enrich G1 or mitotic cell fractions (details on request). For all the synchronization procedures, the predicted cell-cycle compartmentalization was confirmed using FACS analysis.

Immunofluorescence. For immunofluorescence, cells growing on coverslips were fixed with 4% paraformaldehyde, permeabilized, blocked and incubated with antibodies using standard protocols. Alternatively, to maximize clear signals at centrosomes, cells were fixed in cold methanol (−20 °C) for 10 min, blocked and incubated with antibody (see figure legends). Primary antibodies included mouse monoclonal antibody anti-AurA (BD Biosciences; 1:300), rabbit polyclonal anti-phospho-AurA/T288, (Cell Signaling; 1:200), rabbit polyclonal anti-HEF1 1:100, mouse monoclonal antibody anti-HEF1 (14A11) 1:100, rat monoclonal antibody anti-α-tubulin (Abcam; 1:200), rabbit polyclonal anti-γ-tubulin (Abcam; 1:200), mouse monoclonal antibody anti-pericentrin (Covance, Berkeley, CA; 1:250), mouse monoclonal antibody anti-phosphohistone 3 (Upstate), rabbit anti-ninein antibody (1:200), mouse monoclonal antibody anti-c-Nap-1 (BD Biosciences; 1:100), and mouse monoclonal antibody anti-Nek2 (BD Biosciences; 1:100). Secondary antibodies anti-mouse Alexa-488, anti-rabbit Alexa488, anti-mouse Alexa-568, anti-mouse Alexa-633, anti-rabbit Alexa 633, and TOTO-3 dye to stain DNA, were from Invitrogen. Confocal microscopy was performed using a Radiance 2000 laser scanning device coupled to a Nikon Eclipse E800 upright microscope (Carl Zeiss, Thornwood, NY). Statistical analysis of data by one-way ANOVA was performed using GraphPad Instat 3.0 (San Diego, CA).

Kinase assays. For phosphorylation of HEF1 by AurA, an *in vitro* kinase assay was performed using bacterially expressed GST-fused HEF1 derivatives. Histone H3 (Upstate) and H1 (Upstate) were used as positive and negative controls for recombinant AurA (Upstate) phosphorylation, using standard methods except as noted in the Results. In parallel, aliquots without γ-^{32}P(ATP) were processed for SDS–PAGE/Coomassie staining (Invitrogen). GST-pulldown assays used wild-type AurA translated (pCMV-SPORT6-C6) using TnT coupled reticulocyte lysate system (Promega, Madison, WI) mixed with titrated quantities of GST-fused HEF1 derivatives. To analyse HEF1 activation of recombinant, baculovirus-produced AurA, GST–HEF1$_{1-363}$ or GST was titrated into a mixture containing recombinant AurA, immunoprecipitated with anti-AurA, and used for a kinase reaction with γ-^{32}P(ATP) and histone H3 substrate. Aliquots of the reaction mixture were used for SDS–PAGE and western analysis to confirm levels of AurA; phospho-histone H3 was visualized by autoradiography or by phospho-specific antibody. AurA kinase used in Fig. 6c was precipitated from MCF-7-tTA-neo or HEF1 cell lines as well as from MCF-7 cells treated with control or specific oligonucleotide duplex against HEF1 (siHEF1), using anti-AurA antibody (ab1287). The Nek2 kinase assay was performed in standard kinase buffer with addition of an Mg/ATP cocktail (Upstate), with MBP as the substrate.

BIND identifiers. Three BIND identifiers (www.bind.ca) are associated with this manuscript: 334317, 334318 and 334319.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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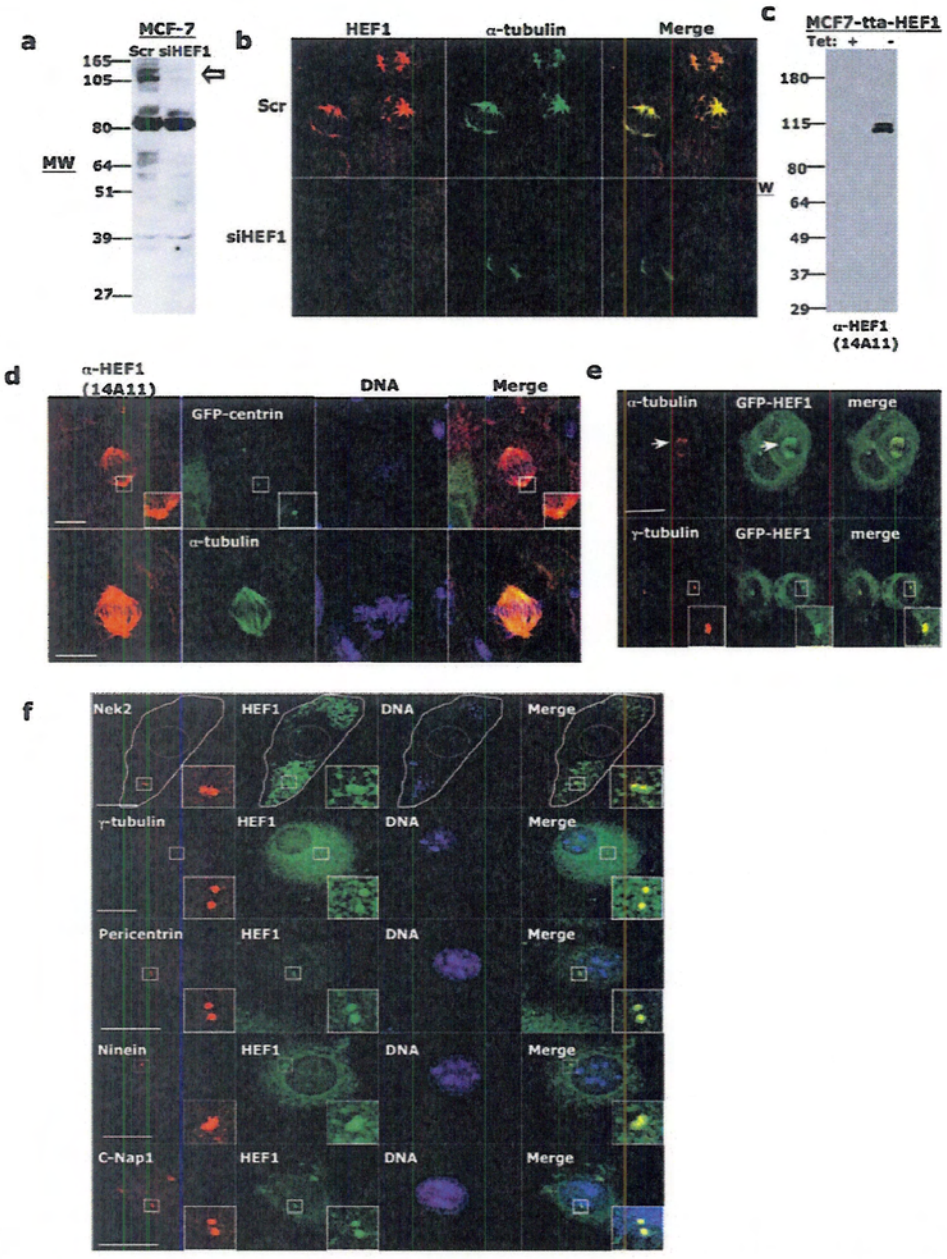

Figure S1 Controls for HEF1 antibody detection at the centrosome. **A**. Western blot with anti-HEF1-SB-R1 antibody, in MCF cells transfected with scrambled (Scr) or HEF1-specific (siHEF1) siRNA. HEF1 indicated with open arrow. **B**. Mitotic cells visualized with anti-HEF1-SB-R1 antibody, following depletion with siHEF1 for 48 hours. A comparable result is obtained with monoclonal anti-HEF1 antibody (clone 14A11) staining (D). **C**. Western blot with 14A11, of cells induced to express HEF1 by tetracycline removal (right lane). **D**. Immunofluorescence profile of MCF7 cells with integrated GFP-centrin (top), or parental MCF7 cells (bottom) stained with new HEF-specific monoclonal antibody 14A11 (red), with GFP-centrin (green), a-tubulin (green), and DNA (blue) also as indicated. The 14A11 antibody also detects HEF1 at focal adhesions (not visible in plane shown here). Scale bars represent 8 μm. **E.** GFP-HEF1 fusion protein confirms HEF1 localization at the centrosome, and at the spindle in mitosis. Cells are co-stained with antibodies to gamma-tubulin (bottom panels) or alpha tubulin (top panels), for visualization of centrosome and spindle. Scale bar represents 6 μm. **F**. Immunofluorescence analysis was used to co-localize HEF1 with the centrosomal marker proteins Nek2, γ-tubulin, pericentrin, ninein and c-Nap-1 (each in red) in methanol-fixed MCF7 cells. HEF1 is shown in green; DNA in blue. Scale bars represent 10 μm. Inset, enlarged view of centrosomes.

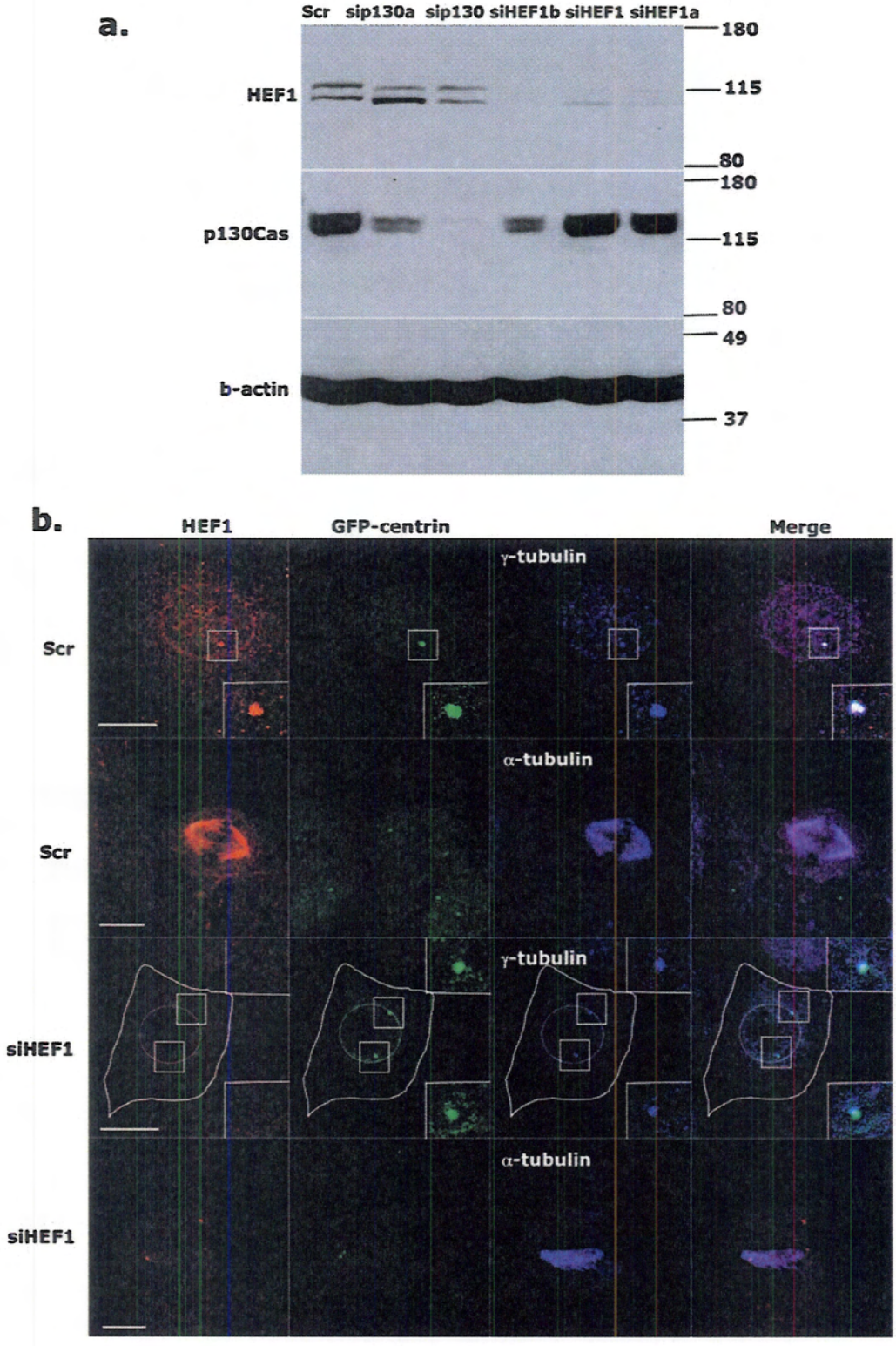

Figure S2 Controls for HEF1 siRNA depletion. **A.** A panel of siRNAs was assessed for degree of depletion of HEF1, p130Cas, or neither (negative control). Of those shown, siHEF1 and siHEF1a were most efficient at specifically depleting HEF1, and were used for studies here. Scr and a GFP-directed siRNA (not shown) depleted neither HEF1 nor p130Cas, and were used as negative controls. sip130 was used in some experiments for comparison to p130Cas depletion. **B.** MCF7 GFP-centrin2 cells were treated with siRNA to HEF1 (siHEF1), or control siRNA (Scr). Immunofluorescence was performed with antibody to HEF1 (red), to γ-tubulin, and to α-tubulin (blue) as indicated; both mitotic and non-mitotic cells are shown. Defects in the centrosomes and mitotic spindle of cells with depleted HEF1 are discussed in the subsequent Results. Scale bars represent 12 μm.

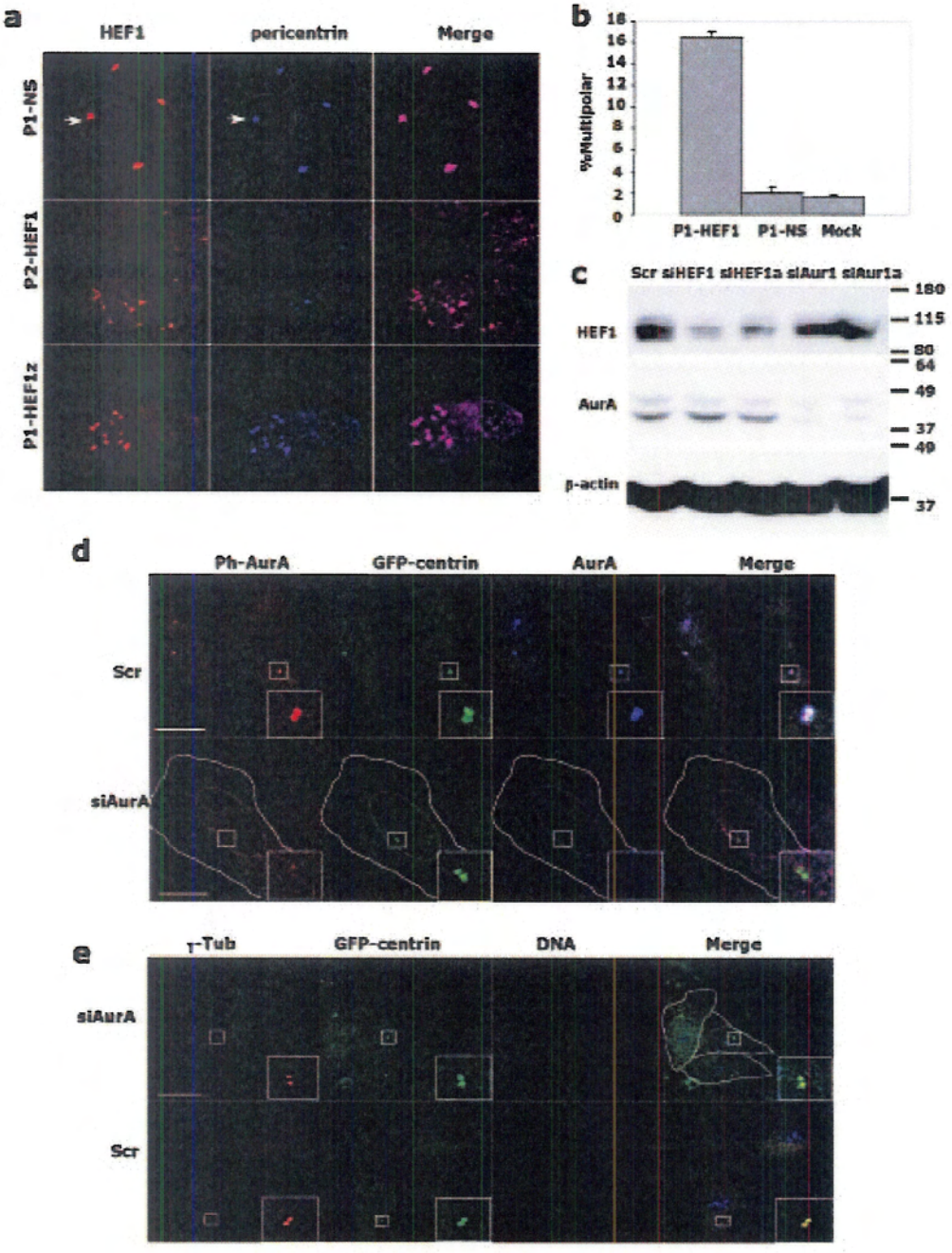

Figure S3 Supernumerary centrosomes and multipolar spindles induced by overexpression of HEF1-stabilizing peptides, and controls for AurA siRNA depletion. **A.** MCF7 cells expressing peptides that stabilize HEF1 (P1-HEF1, P2-HEF1 [14]) or non-specific controls (P1-NS) were fixed and stained with antibody to HEF1 (red) and pericentrin (blue), 48h post infection. **B.** Quantitation of frequency of multipolar spindles among mitotic cells expressing HEF1-targeted or non-specific peptides. **C.** Western analysis with antibody to HEF1 or AurA from HEF1- or AurA-depleted MCF7/GFP-centrin2 cells. **D, E.** MCF7 cells with integrated GFP-centrin2 were treated with siRNA to AurA (siAurA) or control siRNA (Scr). Immunofluorescence was performed with antibody to AurA (B, blue), to phospho-AurA (Ph-AurA) (B, red), and to γ-tubulin (C, red) and DNA (C, blue), as indicated. Scale bars represent 8 μm for **D**, 5μm for **E**.

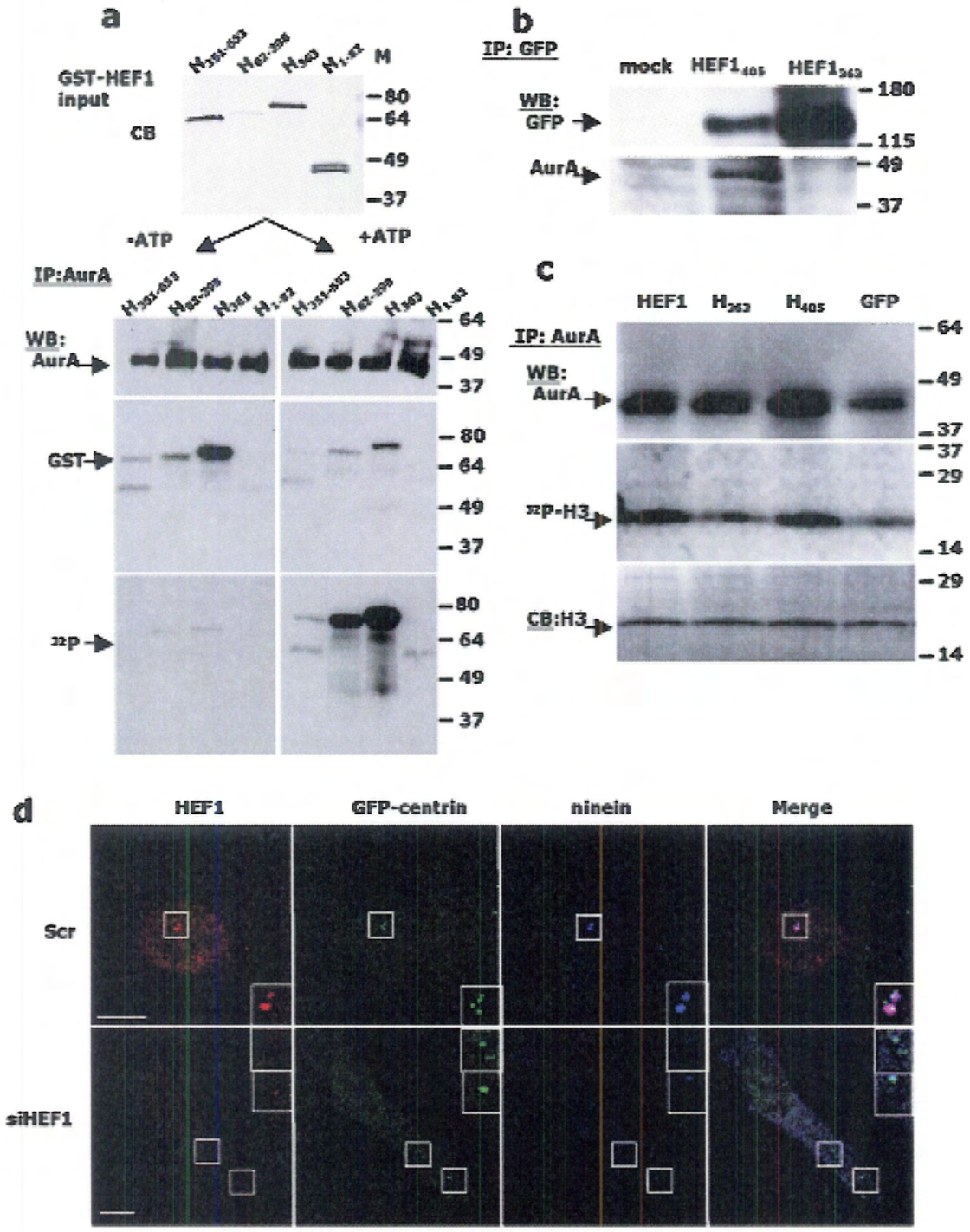

Figure S4 Delineation of the HEF1 domain involved in activation of AurA, and consequences of HEF1 depletion for ninein at the centrosome. **A.** GST-fused HEF1 derivatives (top panel: Coomassie stained gel) was mixed with recombinant AurA. The reaction was split, and incubated in the absence (left) or presence (right) of a source of ATP. The reaction was immunoprecipitated with antibody to AurA, and visualized with antibody to AurA or GST, or by autoradiography (^{32}P, bottom), as indicated. Note, the $HEF1_{82\text{-}398}$ fragment is present at lower levels because it is less stable then other HEF1 domains when produced *in vitro*. **B.** MCF-7 cells were mock-infected or infected with a plasmid encoding GFP-$HEF1_{1\text{-}363}$ or GFP- $HEF1_{1\text{-}405}$, immunoprecipitated with antibody to GFP and Western blot visualization with antibodies to GFP or AurA, as indicated. **C.** Lysates from MCF7 cells infected with lentivirus expressing GFP fused to HEF1, $HEF1_{1\text{-}363}$ (H_{363}), or $HEF1_{1\text{-}405}$ (H_{405}) or GFP only, were used for immunoprecipitation with antibody to AurA. Immunoprecipitates were probed with antibody to AurA (WB:AurA), or used for in vitro kinase analysis with histone H3 as substrate. Total histone H3 visualized with Coomassie Blue (CB:H3) and phospho-histone H3 are shown. Parallel analysis of crude lysates with antibody to GFP (as in Figure 8C) confirmed all lysates contained equivalent amounts of GFP-fusion; result not shown due to space. **D.** MCF7 cells with integrated GFP-centrin were treated with scrambled control siRNA (Scr), or siRNA to HEF1 (siHEF1), and stained for immunofluorescence with antibodies to HEF1 (red) and ninein (blue), as indicated. Scale bars equal 10 μm.